Trastuzumab

DESCRIPTION

WARNING
CARDIOMYOPATHY:

HERCEPTIN® (Trastuzumab) administration can result in the development of ventricular dysfunction and congestive heart failure. l ventricular function should be evaluated in all patients prior to and du treatment with HERCEPTIN. Discontinuation of HERCEPTIN treatme should be strongly considered in patients who develop a clinically significant decrease in left ventricular function. The incidence and severity of cardiac dysfunction was particularly high in patients who received HERCEPTIN in combination with anthracyclines and cyclophosphamide. (See **WARNINGS**)

HERCEPTIN® (Trastuzumab) is a recombinant DNA-derived humanized mc antibody that selectively binds with high affinity in a cell-based assay (Kd = the extracellular domain of the human epidermal growth factor receptor 2 pr HER2.[1,2] The antibody is an IgG_1 kappa that contains human framework re the complementarity-determining regions of a murine antibody (4D5) that bi HER2.

The humanized antibody against HER2 is produced by a mammalian cell (C Hamster Ovary) [CHO] suspension culture in a nutrient medium containing t antibiotic gentamicin. Gentamicin is not detectable in the final product.

HERCEPTIN is a sterile, white to pale yellow, preservative-free lyophilized p intravenous (IV) administration. Each vial of HERCEPTIN contains 440 mg Trastuzumab, 9.9 mg L-histidine HCl, 6.4 mg L-histidine, 400 mgα,α-trehalc dihydrate, and 1.8 mg polysorbate 20, USP. Reconstitution with 20 mL of th Bacteriostatic Water for Injection, (BWFI) USP, containing 1.1% benzyl alco preservative, yields 21 mL of a multi-dose solution containing 21 mg/mL Tra at a pH of approximately 6.

For information on ordering Herceptin Online, please click the "online pharmacy" link on the blue horizontal navigation bar at the top of every page.

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1: J Clin Oncol. 1996 Mar;14(3):737-44.

Comment in:

- J Clin Oncol. 1996 Mar;14(3):697-9.

Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer.

Baselga J, Tripathy D, Mendelsohn J, Baughman S, Benz CC, Dantis L, Sklarin NT, Seidman AD, Hudis CA, Moore J, Rosen PP, Twaddell T, Henderson IC, Norton L.

Department of Medicine, Services of Breast and Gynecological Cancer Medicine and Clinical Immunology, Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, NY, USA.

PURPOSE: Breast cancer frequently overexpresses the product of the HER2 proto-oncogene, a 185-kd growth factor receptor (p185HER2). The recombinant humanized monoclonal antibody (rhuMAb) HER2 has high affinity for p185HER2 and inhibits the growth of breast cancer cells that overexpress HER2. We evaluated the efficacy and toxicity of weekly intravenous administration of rhuMAb HER2 in patients with HER2-overexpressing metastatic breast cancer. PATIENTS AND METHODS: We treated 46 patients with metastatic breast carcinomas that overexpressed HER2. Patients received a loading dose of 250 mg of intravenous rhuMAb HER2, then 10 weekly doses of 100 mg each. Patients with no disease progression at the completion of this treatment period were offered a maintenance phase of 100 mg/wk. RESULTS: Study patients had extensive metastatic disease, and most had received extensive prior anticancer therapy. Adequate pharmacokinetic levels of rhuMAb HER2 were obtained in 90% of the patients. Toxicity was minimal and no antibodies against rhuMAb HER2 were detected in any patients. Objective responses were seen in five of 43 assessable patients, and included one complete remission and four partial remissions (overall response rate, 11.6%; 95% confidence interval, 4.36 to 25.9). Responses were observed in liver, mediastinum, lymph nodes, and chest wall lesions. Minor responses, seen in two patients, and stable

disease, which occurred in 14 patients, lasted for a median of 5.1 months. CONCLUSION: rhuMAb HER2 is well tolerated and clinically active in patients with HER2-overexpressing metastatic breast cancers that had received extensive prior therapy. This is evidence that targeting growth factor receptors can cause regression of human cancer and justifies further evaluation of this agent.

Publication Types:

- Clinical Trial
- Clinical Trial, Phase II

PMID: 8622019 [PubMed - indexed for MEDLINE]

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trastuzumab

Cancer Encyclopedia

THOMSON GALE

Trastuzumab

Definition

Trastuzumab is a humanized monoclonal antibody produced by recombinant DNA technology that binds specifically to the human epidermal growth factor receptor 2 protein (also known as HER2 or neu or c-erb-2) that is found on the cell surface of some cancer tumors, most notably breast cancer. The drug is marketed in the United States under the Herceptin brand name.

Purpose

Trastuzumab is a monoclonal antibody used to treat breast cancers that overexpress the HER2 protein, which occurs in about 25-30% of breast malignancies. By binding the HER2 protein on the tumor cell, the antibody targets it for destruction by the immune system. Based on data gathered in the laboratory, developers believe that trastuzumab triggers cell-mediated means to kill the tumor cells, through the action of natural killer cells and monocytes, two types of white blood cells. As binding of the antibody also slows growth of the tumor, it is theorized that the antibody may also block the interaction of the HER2 protein with a not yet identified growth factor that triggers rapid cell divisions.

Clinical trials have also begun or are soon to begin to test the use of trastuzumab against osteosarcoma, as well as endometrial, colorectal, kidney, pancreatic, prostate, ovarian, salivary gland, lung, and bladder cancers, as all of these tumor types can overexpress the HER2 protein on their surface.

Description

Trastuzumab is a genetically engineered monoclonal antibody. In 1998 it was approved by the FDA as a method of slowing growth of breast cancer tumors that overexpress the HER2 protein on the cell surface. Overexpression or overproduction of the HER2 protein is associated with aggressive disease and increased mortality.

Trastuzumab is approved for use either alone, or in combination with paclitaxel, a drug used for chemotherapeutic treatment of breast cancer. In clinical trials treating patients having breast cancer that has spread beyond the breast (metastatic breast cancer), trastuzumab had an overall response rate of 14%, with 2% having a complete response. When used in combination with paclitaxel treatment, the antibody reduced the risk of death by 24%. Higher expression of the HER2 protein on the tumor surface correlates with an increased chance of response to the drug. Additionally, clinical trials using trastuzumab in the TCH chemotherapy regime (Taxotere, cisplatin or carboplatin, and Herceptin) appears to avoid risk of heart problems (cardiotoxicity) seen with the paclitaxel/Herceptin combination.

Other clinical trials have begun testing the use of trastuzumab with other chemotherapy drugs such as doxorubicin (an antitumor anitbiotic), cyclophosphamide (an alkylating agent that interferes with mitosis and cell division), celecoxib (an aspirin-like drug called a **cyclooxygenase-2 inhibitor**), capecitabine (an antimetabolite that interferes with DNA and RNA growth), and others. Testing the combination of the monoclonal antibody and various cytokines, such as interleukins 2 and 12, is also ongoing. Additionally, doctors are also studying the combination of the antibody with other cancer treatments such as radiation and transplantation with peripheral stem cells.

Most of the trastuzumab sequence is derived from human sequences, while about 10% are from the mouse. The human sequences were derived from the constant domains of human IgG1 (called "constant" because it is essentially the same for all IgG antibodies) and the variable framework regions of a human antibody. These areas do not bind to the epidermal growth factor receptor 2. Using human sequences in this part of the antibody helps to reduce patient immune response to the antibody itself and is called humanization. The actual binding siteof trastuzumab to the receptor is from a mouse anti-HER2 antibody.

Recommended dosage

Trastuzumab is administered intravenously, at a dose of 4 mg/kg for the initial administration, and 2 mg/kg for weekly maintenance until the disease progresses. The antibody can be given for longer periods to maintain tumor shrinkage.

Precautions

Extreme caution should be exercised when using trastuzumab to treat patients with existent heart problems. Also, patients with lung problems have an increased risk of side effects. Because the drug can pass to the fetus through the placenta and is present in breast milk, the drug should be used during pregnancy and nursing only if clearly indicated.

Side effects

The most severe side effects seen with this drug are heart and lung problems, which tend to occur most often in patients with a history of heart or lung disease. The use of anthracyclines and cyclophospamide in combination with trastuzumab also appears to increase these types of side effects.

The most common side effect with trastuzumab are infusion-associated symptoms, usually consisting of fever and chills on first infusion. The symptoms are often mild to moderate in severity and are treated with acetaminophen, diphenhydramine, and/or meperidine. Other common side effects include nausea and vomiting, and pain (in some cases at tumor sites), which occur less often after the first dose. Lowered red blood cell count (anemia), lowered white blood cell count (leukopenia), diarrhea, and infection occur more often in patients receiving Herceptin plus chemotherapy as compared to chemotherapy alone. The severity of these symptoms usually do not result in discontinuation of therapy with Herceptin.

Other less common side effects are headache, abdominal pain, back pain, flu-like symptoms, sinusitis, rhinitis, pharyngitis, fluid retention (edema), insomnia, dizziness and depression.

Interactions

There have been no formal drug interaction studies done for trastuzumab. However, in clinical trials, this drug has

a decreased clearance rate (time of removal from the body) when combined with some chemotherapeutic drugs including paclitaxel.

See Also Monoclonal antibodies

Michelle Johnson, M.S., J.D.

KEY TERMS

Antibody; IgG; Interleukins; Humanization; Monoclonal.

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trastuzumab

Generic Name: trastuzumab

Brand Name: Herceptin

Drug Class And Mechanism: Trastuzumab is an intravenous drug that is used to treat breast cancer that has spread (metastasized). It belongs to a class of drugs called monoclonal antibodies. Other monoclonal antibodies include rituximab (Rituxan) and gemtuzumab ozogamicin (Mylotarg). The cells of tumors have various receptors on their surfaces. Chemicals bind to these receptors and cause changes within the tumor cells. One of the receptors which occurs in about one-third of all breast cancers is called HER2. HER2 is known to control the growth and development of the tumor cells and the production of new tumor cells. If HER2 receptors are present in large numbers on the tumor cells (often referred to as overexpression of HER2), then the tumor cells may multiply and grow quickly. Normally, the immune system produces antibodies that will detect and attack HER2 receptors to slow the growth of tumor cells However, if HER2 is present in large numbers, the immune system may be unable to control HER2. Trastuzumab is a man-made antibody developed using molecular cloning and recombinant DNA technology. Trastuzumab is thought to block the HER2 receptors when there is overexpression and thereby block tumor growth and development. Trastuzumab was approved by the FDA in 1998.

Generic Available: no

Prescription: yes

Preparations: Trastuzumab is available as a powder in a vial containing 440 mg of drug. It must be mixed with a liquid before intravenous injection.

Storage: Trastuzumab should be stored at 2-8°C (36-46°F) and should not be frozen.

Prescribed For: Trastuzumab is used to treat metastatic breast cancer among patients who overexpress HER2. There are two situations in which trastuzumab may be used. In the first, trastuzumab is used as the only drug for patients who already have received one or more regimens of chemotherapy. In the second, trastuzumab is used in

combination with paclitaxel (Taxol) for patients who have not yet received chemotherapy for their metastatic breast cancer.

Dosing: Trastuzumab usually is administered intravenously with an initial dose of 4 mg per kilogram of body weight followed by a weekly dose of 2 mg per kilogram of body weight.

Drug Interactions: There have been no studies of drug interactions with trastuzumab.

Pregnancy: There are not enough studies to draw conclusions about the safety and efficacy of trastuzumab in pregnant women.

Nursing Mothers: Nursing mothers should avoid trastuzumab therapy and not begin nursing for six months after discontinuing the drug. Since trastuzumab is a type of antibody that can be secreted into breast milk and absorbed by the infant, it has the potential for harming nursing infants.

Side Effects: The most common side effects with trastuzumab alone include

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Wikipedia

trastuzumab

Herceptin Logo

Trastuzumab (Herceptin®) is an anti-cancer therapy that acts on the HER2/neu (erbB2) receptor. Herceptin's prir

use is in breast cancer in patients whose tumors overexpress (produce more than the usual amount of) this recept
Trastuzumab is administered either once a week or once every three weeks intravenously for 30 to 90 minutes.

Mechanism of action

Amplification of ErbB2 occurs in 30% of early-stage breast cancers (Bange *et al* 2001). It encodes the transmembra
tyrosine kinase p185-erbB2 glycoprotein. Although the signaling pathways induced by the erbB2 receptor are
incompletely characterized, it is thought that activation of the PI3K/Akt pathway is important. This pathway is no
associated with mitogenic signaling involving the MAPK pathway. In cancer growth producing signals from erbB2 ar
constitutively transmitted, promoting invasion, survival and angiogenesis of cells (Ménard *et al* 2003). Furthermore
overexpression can also confer therapeutic resistance to cancer therapies. Kute et al. (2004) suggest that the prim
mechanism that causes increase in proliferation speed is due to induction of p27Kip1, an inhibitor of cdk2 and of c
proliferation, to remain in the cytoplasm instead of translocation in to the nucleus. This is caused by phosphorylat
Akt.

Herceptin is a monoclonal antibody which binds to the extracellular segment of the erbB2 receptor. Cells treated
Herceptin undergo arrest during the G1 phase of the cell cycle and experience a reduction in proliferation. It has b
suggested that Herceptin induces some of its effect by downregulation of erbB2 leading to disruption of receptor
dimerization and signaling through the downstream PI3K cascade. P27Kip1 is then not phosphorylated and is able t
enter the nucleus and inhibit cdk2 activity, causing cell cycle arrest (Kute *et al* 2004). Also, Herceptin suppresses
angiogenesis by induction of antiangiogenic factors and repression of proangiogenic factors. It is thought that a
contribution to the unregulated growth observed in cancer could be due to proteolytic cleavage of erbB2 that resu
the release of the extracellular domain. Herceptin has been shown to inhibit erbB2 ectodomain cleavage in breast
cancer cells (Albenall *et al* 2003). There may be other undiscovered mechanisms by which Herceptin induces regre
in cancer.

Impact

Herceptin has had a "major impact in the treatment of HER2-positive metastatic breast cancer" (Tan and Swain 20
combination with chemotherapy Herceptin has been shown to increase both survival and response rate in comparis
Herceptin alone (Nahta and Esteva 2003). It is possible to determine the 'erbB2 status' of a tumour, which can be u
to predict efficacy of treatment with Herceptin. If it is determined that a tumour is overexpressing the erbB2 onc
then a patient is eligible for treatment with Herceptin (Yu and Hung 2000). It is surprising that although erbB2 has
affinity for the receptor and the fact that such a high dose can be administered (due to its low toxicity) 70% of pat
do not respond to treatment. In fact resistance is developed rapidly on treatment of virtually all patients. It is
suggested that a mechanism of resistance is the lack p27Kip1 translocation to the nucleus in some strains, enablin
to induce cell proliferation (Kute et al., 2004).

Some recent clincial trials have found trastuzumab reduces the risk of relapse in breast cancer patients by 50% wh
given in the adjuvant setting (i.e. after breast cancer surgery, before the cancer has spread any further) for one y
(Romond *et al* and Piccart-Gebhard *et al* 2005). In one British trial this translated as 9.4% of those on the drug rela
as opposed to the 17.2% of those not on Herceptin.

There has been some recent debate on as to whether these benefits may have been over-stated (e.g., Littlejohn 2

Side effects

One of the significant complications of trastuzumab is its effect on the heart. Trastuzumab is associated with card
dysfunction in 2-7% of cases. The risk of cardiomyopathy is increased when trastuzumab is combined with anthracy
chemotherapy (which itself is associated with cardiac toxicity).

History

The biotech company Genentech gained FDA approval for trastuzumab in September 1998. The drug was jointly

developed by that company, where the antibody was first discovered by scientists that included Dr Axel Ullrich, ar Jonsson Cancer Center at UCLA, where Dr Dennis Slamon subsequently worked further on trastuzumab's developme

In the clinical trials leading up to trastuzumab's approval 42% of patients taking trastuzumab in combination with t chemotherapy drug paclitaxel had significant responses. The comparable rate for the taxane alone was only 16%.

References

- Albanell J, Codony J, Rovira A, Mellado B, Gascon P. (2003). "Mechanism of action of anti-HER2 monoclonal antibodies: scientific update on trastuzumab and 2C4". *Advances in Experimental Medicine and Biology* **532** 268. PMID 12908564.
- Bange J, Zwick E, Ullrich A. (2001). "Molecular targets for breast cancer therapy and prevention". *Nature Medicine* **7**: 548-552. PMID 11329054.
- Kute T, Lack CM, Willingham M, Bishwokama B, Williams H, Barrett K, Mitchell T, Vaughn JP (2004). "Development of Herceptin resistance in breast cancer cells". *Cytometry* **57A**: 86-93. PMID 14750129.
- Littlejohns P. (2006). "Trastuzumab for early breast cancer: evolution or revolution?". *Lancet Oncology* **7** (1 23. PMID: 16408378
- Ménard S, Pupa SM, Campiglio M, Tagliabue E (2003). "Biologic and therapeutic role of HER2 in cancer". *Onc* **22**: 6570-6578. PMID 14528282.
- Nahta R, Esteva1 FJ (2003). "HER-2-Targeted Therapy - Lessons Learned and Future Directions". *Clinical Car Research* **9**: 5078-5048. PMID 14613984.
- Piccart-Gebhart MJ, Procter M, Leyland-Jones B, et al. (2005). "Trastuzumab after adjuvant chemotherapy HER2-positive breast cancer". *New England Journal of Medicine* **353**: 1659-1672. PMID 16236737.
- Romond EH, Perez EA, Bryant J, et al. (2005). "Trastuzumab plus adjuvant chemotherapy for operable HER2 positive breast cancer". *New England Journal of Medicine* **353**: 1673-1684. PMID 16236738.
- Tan AR, Swain SM (2002). "Ongoing adjuvant trials with trastuzumab in breast cancer". *Seminars in Oncolog* (5 Suppl 16): 54-64. PMID 14613027.
- Yu D, Hung M (2000). "Overexpression of ErbB2 in cancer and ErbB2-targeting strategies". *Oncogene* **19**: 611 6121. PMID 11156524.

Further reading

- Bazell, Robert. *Her-2: the making of Herceptin, a revolutionary treatment for breast cancer*. Random Hous 1998. 214 pages. ISBN 067945702X.
- The Guardian. *The selling of a wonder drug*. 29th March 2006

External links

- Herceptin (manufacturer's website)
- Trastuzumab (patient information)

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Volume 2, Supplement 1 *for health professionals who care for cancer patients* Nov 1999

INSIDE THIS ISSUE

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BENEFIT DRUG LIST

The following new program has been funded by the Provincial Systemic Therapy Program effective 22 November 1999:

Palliative therapy for metastatic breast cancer using trastuzumab (Herceptin®) and paclitaxel (Taxol®) as first-line treatment for recurrent breast cancer refractory to anthracycline adjuvant chemotherapy

Trastuzumab is now approved as a Class II drug on the benefit list when used in combination with paclitaxel. A Class II form must be completed and submitted to the Provincial Systemic Therapy Program before the drug will be dispensed at a radiation cancer centre or reimbursed to a community hospital. Use of trastuzumab as a single agent therapy will continue to require approval under "Undesignated Indication".

Susan O'Reilly, MB, FRCPC
Provincial Systemic Program Leader

PROTOCOL UPDATE

Protocol codes for treatments requiring "Undesignated Indication" approval prior to use are prefixed with the letter U.

- INDEX to BCCA Protocol Summaries revised monthly (includes tumour group, protocol code, indication, drugs, last revision date and version)
- BRAVTRAP new (replacing UBRAVTRAP): Palliative therapy for metastatic breast cancer using trastuzumab and paclitaxel as first-line treatment for recurrent breast cancer refractory to anthracycline adjuvant chemotherapy

FOCUS ON TRASTUZUMAB (HERCEPTIN®)

Trastuzumab (Herceptin®) is an antibody against HER2, an oncogene overexpressed in some cancer cells. HER2 overexpression occurs in 20-30% of breast cancer patients.

Trastuzumab in combination with paclitaxel is included on the BCCA Benefit Drug List for patients with metastatic breast cancer who (1) substantially overexpressed HER2 and (2) have relapsed within 12 months of anthracycline-containing adjuvant chemotherapy (see protocol BRAVTRAP). A Class II form must be submitted.

Trastuzumab and Chemotherapy

A randomised multinational controlled Phase III trial studied the addition of trastuzumab to chemotherapy in women with metastatic breast cancer that overexpressed HER2. Four hundred and sixty-nine patients received either (1) doxorubicin-cyclophosphamide (AC) or (2) paclitaxel for those patients that had previously received anthracyclines.[1] As well, half of the patients were randomised to receive weekly trastuzumab.

The addition of trastuzumab to chemotherapy significantly improved response rates and time to disease progression compared to chemotherapy alone. The response rate for paclitaxel plus trastuzumab was 57% (vs. 25% with paclitaxel alone) and time to disease progression was 7.1 months (vs. 4.2 months with paclitaxel alone). Although the same improvement was seen with the

addition of trastuzumab to AC, excessive cardiotoxicity in this group precludes the use of this combination in clinical practice.

Cardiotoxicity

There was a 2-4% incidence of cardiotoxicity using trastuzumab with paclitaxel.[2,3] The mechanism of the cardiotoxicity is not clear but appears to be related to previous anthracycline exposure. For patients with equivocal cardiac status, a MUGA scan or echocardiogram should be done prior to treatment. Only patients with a normal left ventricular ejection fraction should be treated with trastuzumab.

Administration

Trastuzumab is given intravenously using a loading dose of 4 mg/kg followed by weekly maintenance doses of 2 mg/kg. The first trastuzumab dose is given in 250 mL NS over 90 minutes. If well tolerated, subsequent trastuzumab doses can be given over 30 minutes. The first paclitaxel dose is given the day following the first trastuzumab dose, but if well tolerated both drugs can subsequently be scheduled for the same day.

Trastuzumab is given weekly whereas paclitaxel is given every 3 weeks. If there is no response after 2 cycles of paclitaxel, the treatment should be discontinued. Otherwise the planned treatment duration for paclitaxel is 6 doses in a responding patient. At this time the optimal duration of trastuzumab therapy is unknown so it may be continued until progression or toxicity in a responding patient. Continued use of trastuzumab as single agent therapy beyond 6 cycles would require an approval for "Undesignated Indication". Because of the risk of cardiotoxicity, the patient must be carefully monitored for both response and toxicity.

Trastuzumab Infusion-Associated Symptoms

Chills and fever occur in 40% of patients during the first trastuzumab infusion but are infrequent with subsequent infusions. Other signs and symptoms may include nausea, vomiting, pain (sometimes at tumour sites), rigors, headache, dizziness, dyspnea, hypotension, rash and asthenia. Symptoms may be treated with acetaminophen, diphenhydramine and meperidine with or without an infusion rate reduction.

R. O'Brien PharmD, BCCA Drug Information Pharmacist
Reviewed by K. Gelmon, MD, BCCA Medical Oncologist

References

1. Slamon D et al. Proc Am Soc Clin Oncol 1998;17:98a.
2. Herceptin® Monograph, Hoffmann-La Roche August 1999.
3. Norton L et al. Proc Am Soc Clin Oncol 1999;18:127a.

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Mário de Lemos, Update Editor	(604)-877-6098 Ext 2288	mdelemos@bccancer.bc.ca
Francis Hu, CON Pharmacist	(604)-877-6098 Ext 2515	francish@bccancer.bc.ca

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❑		Class 2 Form (01 Nov 99)
❑		Undesignated Drug Request Form (01 Sep 99)

CLINICAL THERAPEUTICS®/VOL. 21, NO. 2, 1999

New Drugs

Trastuzumab, a Recombinant DNA–Derived Humanized Monoclonal Antibody, a Novel Agent for the Treatment of Metastatic Breast Cancer

Marvin M. Goldenberg, PhD
Mount Sinai NYU Health, New York, New York

ABSTRACT

Amplification of the human epidermal growth factor receptor 2 protein (HER2) in primary breast carcinomas has been shown to correlate with poor clinical prognosis for certain patients. Trastuzumab (Herceptin®, Genentech, Inc., South San Francisco, California) is a highly purified recombinant DNA–derived humanized monoclonal immunoglobulin G1 kappa antibody that binds with high affinity and specificity to the extracellular domain of the HER2 receptor. In vitro and in vivo preclinical studies have shown that administration of trastuzumab alone or in combination with paclitaxel or carboplatin significantly inhibits the growth of breast tumor–derived cell lines that overexpress the *HER2* gene product. At therapeutic doses in breast cancer patients, the mean half-life of trastuzumab is 5.8 days. Trastuzumab serum concentrations reach steady state with mean trough and peak concentrations of 79 μg/mL and 123 μg/mL, respectively. In a 222-patient, single-arm clinical study, treatment with a loading dose of trastuzumab 4 mg/kg administered IV followed by weekly IV doses of 2 mg/kg produced an overall response rate of 14% (2% complete remission and 12% partial remission). The beneficial effects were greatest in patients with the greatest degree (3+) of HER2 protein overexpression. In another clinical study, 469 women with metastatic breast carcinoma were randomized to a paclitaxel or anthracycline-plus-cyclophosphamide regimen with or without trastuzumab. The overall response rate was significantly greater in the trastuzumab-plus-chemotherapy group than in the chemotherapy-alone cohort. The magnitude of observed effects was greatest with pacli-

0149-2918/99/$19.00

taxel plus trastuzumab. The most common adverse effects attributed to trastuzumab in clinical studies were fever and chills, pain, asthenia, nausea, vomiting, increased cough, diarrhea, headache, dyspnea, infection, rhinitis, and insomnia. Trastuzumab in combination with chemotherapy can lead to cardiotoxicity, leukopenia, anemia, diarrhea, abdominal pain, and infection. Trastuzumab has been approved by the US Food and Drug Administration as a single agent for the treatment of patients who have metastatic breast cancer involving overexpression of the HER2 protein and who have received 1 or more chemotherapy regimens; in combination with paclitaxel, it has been approved for the treatment of such patients who have not received chemotherapy. ***Key words:*** trastuzumab, HER2 overexpression, metastatic breast cancer, rhuMabHER2.

INTRODUCTION

Based on data in the Surveillance, Epidemiology, and End Results database of the National Cancer Institute, the age-adjusted incidence of invasive breast cancer in white and black women for the years 1990 and 1995 was 114.5 and 100.5 cases per 100,000 persons, respectively; the mortality rate was 26 and 31.5 cases per 100,000 persons, respectively.[1] It was estimated that breast cancer (excluding breast carcinoma in situ) would be newly diagnosed in 178,700 women and be the reported cause of death in 43,500 women in 1998.[2] In 1994, stage IV or metastatic breast cancer constituted 3.6% and 6.2% of all breast cancer types among white and black women, respectively.[3] It has been estimated that over 80,000 women per year will develop metastatic or refractory breast cancer, with rates of metastatic disease having remained constant in white women between 1973 and 1993.[4,5]

Growth factors and their receptors play pivotal roles in the regulation of cell growth and differentiation.[6] Malignancy arises from a stepwise progression of genetic events that often includes unregulated expression of growth factor receptors or elements of their signaling pathways.[6,7] Overexpression, or amplification, of the human epidermal growth factor receptor (EGFR) 2 protein (HER2), which is correlated with poor clinical outcome in patients with breast cancer, is believed to result from gene amplification.[8] This protein is located on the cell's surface, where it interacts with growth factors. When the HER2 protein is overexpressed, the cells divide, grow, and multiply at a faster rate than normal, contributing to the development of cancer. When both node-negative and node-positive breast cancers were reviewed, a significant difference in 5-year survival was found between primary breast cancers that overexpressed HER2 compared with those that did not.[9] Studies in breast cancer patients have shown that 25% to 30% of breast cancers overexpress the *HER2* gene.[10]

The *HER2* gene (also known as *neu* and *c-erbB-2*) encodes a 185-kd transmembrane/kinase receptor, designated p185HER2, that has partial homology with the other members of the EGFR family.[11–13] Antibodies directed against HER2 can inhibit the growth of tumor xenografts and transformed cells that express high concentrations of this receptor.[14,15] The murine monoclonal antibody mumAB4D5, directed against human p185HER2, has been shown to specifically inhibit proliferation of human tumor cells overexpressing this receptor.[16] Amplification or overexpression of HER2 is associated with multiple human malignancies, especially breast can-

cer,[17] and with more rapid cancer progression and shortened survival.[10] A similar but less frequent amplification of the *HER2/c-erbB-2* gene has been described in gastric adenocarcinoma.[18]

DESCRIPTION OF TRASTUZUMAB

A recombinant humanized anti-HER2 antibody, trastuzumab (Herceptin®, Genentech, Inc., South San Francisco, California) was constructed and developed to determine whether its use, either alone or in combination with chemotherapeutic agents, could reduce the progression of malignancy in women with advanced breast cancer. Assays were performed both in vitro and in animal models to find whether the recombinant human monoclonal antibody inhibited the proliferation of tumor cells overexpressing HER2. The agent's safety was also a critical concern of these studies.

Trastuzumab is a highly purified recombinant DNA–derived humanized monoclonal immunoglobulin G1 kappa antibody that in a cell-based assay (kd = 5 nmol), binds selectively and with high affinity to the extracellular domain of the human EGFR2 protein, HER2.[10,17] It is produced by mammalian cell culture using Chinese hamster ovary suspension culture in a nutrient medium containing the antibiotic gentamicin. The antibiotic is not detectable in the final product. The approximate weight of the antibody is 148 kd.

Trastuzumab binds with high affinity and specificity to the extracellular domain of the HER2 receptor that is overexpressed in some breast cancer cells. The antibody contains human framework regions, with the complementarity-determining regions of a murine antibody (4D5) that binds to HER2.[15]

PRECLINICAL STUDIES

In 1 study,[19] SKBR3 human breast tumor cells overexpressing the *HER2/c-erbB-2* gene or A431 human squamous carcinoma cells overexpressing the *EGFR* gene were grown in flasks. The cells were detached from the flasks by treatment with 25 mmol/L ethylenediamine–tetraacetic acid/0.15 mol/L sodium chloride, collected by low-speed centrifugation, and suspended at 1×10^6 cells/mL in phosphate buffered saline/1% fetal bovine serum. Each cell line (1 mL) was incubated with 10 μg of either anti-HER2/c-erbB-2 monoclonal antibody (4D5) or a control antibody recognizing the hepatitis B surface antigen. The monoclonal antibody 4D5 was bound to the surface cells of the human tumor cell line expressing p185HER2, as measured by fluorescence-activated cell sorting. There was a 160-fold increase in cellular fluorescence compared with a control monoclonal antibody when 4D5 was added to SKBR3 breast adenocarcinoma cells. This cell line contains an amplified *HER2/c-erbB-2* gene and expresses high concentrations of p185HER2.[19,20] In contrast, the squamous carcinoma cell line A431, which expresses about 2×10^6 EGFR per cell[21] but only low concentrations of p185HER2, exhibited only a twofold increase in fluorescence with 4D5 compared with a control monoclonal antibody.

Most anti-HER2/c-erbB-2 monoclonal antibodies that recognize the extracellular domain inhibited the growth of SKBR3 cells in this study.[19] Maximum inhibition was obtained with monoclonal antibody 4D5, which inhibited cellular proliferation by 56%. The control antibodies had no significant effect on cell growth.

In another study,[16] the humanized monoclonal antibody 4D5-8 was found to

bind to p185HER2 with high affinity (250 times control) and to prevent the proliferation of the human mammary adenocarcinoma cell line SKBR3. The monoclonal antibody also promoted antibody-dependent cellular toxicity against SKBR3 tumor cells in the presence of human effector cells but was not effective in directing the killing of normal (WI-38) cells, which express p185HER2 at much lower concentrations. This finding predicted effective treatment of human cancers that overexpress p185HER2 with human antibody 4D5-8.

The results of these 2 studies indicate that trastuzumab has a higher affinity for p185HER2 (kd = 0.1 nmol) than does the murine Mab 4D5 and has a cytostatic growth-inhibitory effect against breast cancer cells overexpressing HER2. An additional in vitro study[22] suggests that the growth of those breast cancer cell lines having the highest basal concentration of p185HER2 is most inhibited by the anti-p185HER2 antibody.

In another in vitro study,[23] a significant synergistic suppression of cell proliferation was observed between 4D5 antibody and cisplatin in SKBR3 breast carcinoma cells overexpressing the *HER2/neu* gene ($P < 0.001$). Such synergistic activity was also noted when 4D5 was added to SKBR3 cells in combination with carboplatin ($P < 0.001$). To confirm the relative receptor-dependent specificity of this phenomenon, C13pRV-CON cells that did not overexpress the *HER2/neu* proto-oncogene were treated with identical antibody/drug combinations, and no apparent synergistic decrease in cell growth was observed. The synergistic effect of the combination of 4D5 and cisplatin was confirmed in vivo in tumor-bearing mice, where significant and marked inhibition of tumor growth was observed that exceeded the effect of either agent alone ($P < 0.005$).

In a study of tumor growth in athymic nude mice,[24] treatment with rhuMabHER2 0.1 to 30 mg/kg intraperitoneally twice weekly plus paclitaxel 5 to 10 mg/kg IV on days 1 and 4 significantly enhanced inhibition of tumor growth (93%, $P = 0.006$) compared with either agent administered alone. In addition, tumor growth inhibition at 5 weeks was significantly superior in the group treated with the monoclonal antibody plus paclitaxel compared with paclitaxel alone ($P = 0.016$) but not rhuMabHER2 alone ($P = 0.4$). Therapy combining the antibody with doxorubicin inhibited growth by 70% versus control-treated mice ($P = 0.04$) but was not statistically superior to doxorubicin alone ($P = 0.16$) or the antibody alone ($P = 0.59$).

PHARMACOKINETICS

The pharmacokinetic properties of trastuzumab have been studied in patients with metastatic breast cancer.[25] Short-duration IV infusions of 10 to 500 mg once weekly showed dose-response kinetics. That is, mean half-life increased and clearance decreased with increasing doses. The half-life averaged 1.7 and 12 days at the 10- and 500-mg doses, respectively. The volume of distribution was approximately equal to that of serum volume (44 mL/kg). At the highest weekly dose (500 mg) studied, mean peak serum concentrations were 377 μg/mL.

In studies of trastuzumab using a loading dose of 4 mg/kg followed by a weekly maintenance dose of 2 mg/kg, the mean half-life was 5.8 days (range, 1 to 32 days).[26] Between weeks 16 and 32, trastuzumab serum concentrations reached

steady-state with mean trough and peak concentrations of approximately 79 and 123 μg/mL, respectively.

Detectable concentrations of the circulating extracellular domain of the HER2 receptor (shed antigen) were found in the serum of some patients with tumors overexpressing HER2. Determination of shed antigen in baseline serum samples revealed that 64% of patients had detectable shed antigen at concentrations ranging as high as 1.88 μg/mL. Patients with higher baseline concentrations of shed antigen were more likely to have lower serum trough concentrations of trastuzumab. However, with weekly dosing, most patients with elevated concentrations of shed antigen achieved target serum concentrations of trastuzumab by week 6.[25]

CLINICAL EXPERIENCE

A study was conducted in 46 patients with metastatic breast cancer that overexpressed HER2.[27] Patients received a loading dose of 250 mg trastuzumab IV over a 90-minute period (day 0), followed by one 100-mg dose for 10 weeks beginning on day 7. At the conclusion of the treatment period, patients with stable disease or a minor, partial, or complete response were entered into a maintenance phase consisting of weekly trastuzumab administration until disease progression.

All patients had measurable disease, had a Karnovsky performance status of ≥60% (required occasional help carrying out normal activities), and retained hematologic, hepatic, renal, and pulmonary function. Chemotherapy or additive hormone therapy was not permitted within 3 weeks before study entry (6 weeks for mitomycin or nitrosoureas). Tumor expression of HER2 was determined by immunohistochemical analysis.[8,10]

Tumor response was ascertained at the completion of the initial 11-week treatment period. Complete response was defined as the disappearance of all radiographically or visually apparent tumors; partial response as a ≥50% reduction in the sum of the products of the perpendicular diameters of all measurable lesions; minimal response as a ≥25% and <50% reduction in the diameters of all measurable lesions; stable disease as no change ≥25% in the size of any measurable lesion; and progressive disease as a >25% increase in any measurable lesion or the appearance of any new lesion. To be considered responders, patients had to have achieved at least stability of bone lesions. Time to tumor progression was calculated from the beginning of therapy to progression.

Forty-three patients were assessable for treatment response on day 77. Three patients were not assessable for response: 1 had a bacteremic infection of an IV catheter that required prolonged antibiotic administration, precluding treatment with trastuzumab; 1 patient discontinued treatment for personal reasons; and 1 patient died of congestive heart failure associated with prior doxorubicin treatment. The overall response rate to trastuzumab (complete plus partial responses) was 11.6% (95% confidence interval, 4.36 to 25.9) in the 43 patients. Two patients had a minimal response, and 14 patients had stable disease at day 77. These patients entered a maintenance phase consisting of weekly antibody administration until progression of disease. The median time to progression for patients with either minimal or stable disease was 5.1 months.

An additional patient had a >50% reduction in the size of the metastatic le-

sions on her mediastinum and chest wall after 2 weeks of treatment. Although the duration of the response was >4 weeks, by evaluation day 77 the lesion had begun to regrow from the size reached at its maximal response to therapy. According to the protocol, this patient was considered not to have had a response to treatment but rather to have had progression of disease. Thus 37% of the patients achieved a minimal response or stable disease with trastuzumab treatment.

Therapy with trastuzumab was well tolerated. Of 768 administrations of trastuzumab, only 11 events occurred that were considered related to use of the antibody. Fever and chills occurred on 5 occasions after the first administration of trastuzumab. The fever lasted <8 hours in all cases and did not recur on subsequent administrations of the antibody. Three patients experienced chest pain in areas of tumor involvement shortly after infusion of the first dose of trastuzumab. The pain did not recur on successive administrations of the antibody. None of the patients whose cancer regression met the formal criteria for complete or partial response had pain at a tumor site after administration of trastuzumab.

The activity of trastuzumab was assessed in a phase II efficacy trial in 222 women.[27] This was a multicenter, open-label, single-agent, single-arm study in patients with metastatic breast cancer that overexpressed HER2. Of the 222 patients, 66% had received prior adjuvant chemotherapy, 68% had received 2 prior chemotherapy regimens for metastatic disease, and 25% had received prior myeloablative treatment with hematopoietic rescue. Patients were treated with an IV loading dose of 4 mg/kg, followed by weekly doses of trastuzumab at 2 mg/kg IV. Nine patients did not receive trastuzumab. The overall response rate (complete plus partial responses), as determined by an independent response-evaluation committee, was 14%, with a 2% complete response rate and a 12% partial response rate. The overall response rate in trastuzumab-treated patients was 21%. Major responses were observed in lesions of the liver, mediastinum, lymph nodes, and chest wall. The median survival was 13 months (Kaplan-Meier estimate). Two patients discontinued treatment because of adverse effects. Reduction in cardiac ejection fraction was observed in 9 patients, of whom 6 were symptomatic; all 9 had had either prior anthracycline therapy or a significant cardiac history at study entry. One patient died of ventricular arrhythmia.

The degree of HER2 overexpression was a predictor of treatment effect in this study. Tumor specimens were subjected to a research-use-only immunohistochemical assay and scored from 0 to 3+, with 3+ indicating the strongest positivity. Only patients with 2+ or 3+ tumors (about 33% of those screened) were eligible for study entry. Data from the efficacy trial[27] suggested that beneficial treatment effects were largely limited to patients with the highest level (3+) of HER2 protein overexpression.

The phase III study was a multinational, randomized, open-label, controlled study that assessed the safety and effectiveness of adding trastuzumab to first-line chemotherapy regimens.[28] The primary end point of the study was time to disease progression. A total of 469 women with metastatic breast carcinoma were randomized to treatment with either paclitaxel or anthracycline plus cyclophosphamide with or without trastuzumab. Eligible women were >18 years of age, had a Karnovsky performance status of

≥60%, and had received no previous cytotoxic chemotherapy for metastatic disease. Women who had received prior hormone therapy or radiotherapy in an adjuvant or metastatic setting were eligible, as were women who had received cytotoxic chemotherapy in the adjuvant setting. Patients who had received prior adjuvant anthracycline therapy were assigned to paclitaxel therapy, and patients who had not had prior adjuvant anthracycline therapy were assigned to the anthracycline treatment group. Trastuzumab was administered IV as a 4-mg/kg loading dose on day 0, followed by weekly IV infusions of 2 mg/kg until disease progression. Each chemotherapeutic regimen was administered every 3 weeks for 6 cycles.

Patients who were randomized to trastuzumab plus chemotherapy benefited measurably in terms of time to disease progression, overall response rate, median duration of response, and 1-year survival compared with patients treated with chemotherapy alone. As determined by the independent response assessment committee, the median time to disease progression was 7.2 months in the trastuzumab-plus-chemotherapy cohort, compared with 4.5 months in the chemotherapy-alone cohort. The difference in overall time to disease progression between treatment groups was statistically significant ($P < 0.0001$). The overall response rate was 45% in the trastuzumab-plus-chemotherapy group, compared with 29% in the chemotherapy-alone group ($P < 0.001$). The magnitude of effects was greatest in the paclitaxel subgroup.

Both trastuzumab cohorts in the phase III trial[28] had an increased incidence of cardiac dysfunction compared with either anthracycline plus cyclophosphamide alone or paclitaxel alone. The incidence and severity of cardiac dysfunction were particularly high in those patients who received trastuzumab in combination with anthracycline therapy. The reported incidence of any cardiac dysfunction (which could include dyspnea, increased cough, paroxysmal nocturnal dyspnea, peripheral edema, S3 gallop, and reduced ejection fraction) was 28% in patients treated with trastuzumab plus anthracycline and 7% in patients treated with anthracycline alone. Patients randomized to trastuzumab plus paclitaxel had a reported 11% incidence of cardiac dysfunction, compared with 1% with paclitaxel alone. Nineteen percent of patients in the trastuzumab-plus-anthracycline cohort developed congestive heart failure of class III or IV severity.

OVERALL SAFETY

The most common adverse effects attributed to trastuzumab in the clinical trials were fever and chills and other constitutional symptoms, most often infusion related. Other adverse effects reported included pain, asthenia, nausea, vomiting, increased cough, diarrhea, headache, dyspnea, infection, rhinitis, and insomnia. Mild-to-moderate diarrhea was experienced by 25% of patients.

In the randomized, controlled clinical trials, the incidence of the following adverse events was higher in patients receiving trastuzumab in combination with chemotherapy than in patients receiving chemotherapy alone: cardiotoxicity, leukopenia, anemia, diarrhea, abdominal pain, and infection. The majority of cytopenic events were mild or moderate and reversible, and none resulted in discontinuation of trastuzumab therapy.

During the first infusion of trastuzumab, a symptom complex most commonly con-

sisting of chills and/or fever was observed in about 40% of patients. The symptoms were usually of mild-to-moderate severity and were treated with acetaminophen, diphenhydramine, or meperidine. Overall, discontinuation of trastuzumab treatment was infrequent. Other signs and symptoms after the first infusion included nausea, vomiting, pain, rigors, headache, dizziness, dyspnea, hypotension, rash, and asthenia. Symptoms occurred infrequently with subsequent trastuzumab infusions.

PRESCRIBING INFORMATION

Treatment may be carried out in an outpatient setting by administering a 4-mg/kg trastuzumab loading dose by IV infusion over 90 minutes and subsequent weekly IV doses of 2 mg/kg administered over 30 minutes.

As a single agent, trastuzumab is indicated for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein and who have received 1 or more chemotherapy regimens for their metastatic disease. Trastuzumab is indicated in combination with paclitaxel for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein and who have not received chemotherapy for their metastatic disease.

DISCUSSION AND CONCLUSIONS

Trastuzumab is the first biologic agent or recombinant humanized monoclonal antibody designed to selectively target the extracellular HER2 receptor. The results of preclinical and clinical studies indicate that an anti-EGFR-directed strategy is useful in the treatment of advanced metastatic breast cancer. The US Food and Drug Administration has approved trastuzumab for marketing as a single agent or in combination with paclitaxel in patients whose tumors overexpress HER2.

The use of trastuzumab in combination with chemotherapeutic agents is not without substantial risk (ie, the possibility of developing clinically significant congestive heart failure, cardiac dysfunction, and ventricular dysfunction). Therefore, candidates for trastuzumab therapy should undergo a thorough baseline cardiac assessment (eg, electrocardiogram, echocardiogram, and multigated angiogram). Patients receiving trastuzumab should be monitored frequently for deteriorating cardiac function. The probability of cardiac dysfunction appears to be greatest in patients receiving trastuzumab concurrently with anthracycline and cyclophosphamide therapy. Discontinuation of trastuzumab therapy should be considered in patients with signs and symptoms of clinically significant congestive heart failure, as well as those with a significant decrease in left ventricular function.

Amplification of the *HER2* gene is a predictor of both relapse and survival in patients with node-positive breast cancer and is superior to all other prognosticators with the exception of lymph-node positivity.[8] The fact that the *HER2* gene is altered in 25% to 30% of these cases and that alteration is associated with poor patient outcome indicates that HER2 may play a role in the pathogenesis of a significant number of human tumors. However, this does not preclude the possibility that other genes or genetic elements may be of importance in these diseases. The *HER2* gene should be a focus of attention in the diagnosis and treatment of human breast cancer.

Although trastuzumab has been approved, additional studies are needed to

determine its efficacy in combination with chemotherapy compared with chemotherapy alone in patients with 2+ HER2 overexpression (weakly positive). Additional studies are also needed to predict and diminish the risk of trastuzumab-induced cardiotoxicity.

Address correspondence to: Marvin M. Goldenberg, PhD, Mount Sinai NYU Health, 1 Gustave Levy Place, New York, NY 10029.

REFERENCES

1. Wingo PA, Ries LA, Rosenberg HM, et al. Cancer incidence and mortality, 1973–1995. *Cancer.* 1998;82:1197–1207.

2. Landis SH, Murray T, Bolden S, et al. Cancer statistics, 1998. *Ca Cancer J Clin.* 1998;48:6–29.

3. Ries LA, Kosary CL, Hankey BF, et al. SEER cancer statistics review, 1973–1994. NIH Publication no. 97-2789. Bethesda, Md: National Cancer Institute; 1997:1–55.

4. Honig SL. Treatment of metastatic disease. In: Harris JR, ed. *Diseases of the Breast.* Philadelphia, Pa: Lippincott-Raven; 1996: 669–734.

5. Chu KC, Tarone RE, Kessler LG, et al. Recent trends in U.S. breast cancer incidence, survival, and mortality rates. *J Natl Cancer Inst.* 1996;88:1571–1579.

6. Aaronson SA. Growth factors and cancer. *Science.* 1991;254:1146–1153.

7. Harris JR, Lippman ME, Veronesi U, et al. Breast cancer. *NEJM.* 1992;327:473–480.

8. Slamon DJ, Clark GM, Wong SG, et al. Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science.* 1987; 235:177–182.

9. Sjogren S, Inganas M, Lindgren A, et al. Prognostic and predictive value of c-erbB-2 overexpression in primary breast cancer, alone and in combination with other prognostic markers. *J Clin Oncol.* 1998;16: 462–469.

10. Slamon DJ, Godolphin W, Jones LA, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science.* 1989;244:707–712.

11. Hynes NE, Stern DF. The biology of erb-2/neu/HER-2 and its role in cancer. *Biochim Biophys Acta.* 1994;1198:165–184.

12. Kraus MH, Issing W, Miki T, et al. Isolation and characterization of ErB3, a third member of the erb/epidermal growth factor receptor family: Evidence for overexpression in a subset of human mammary tumors. *Proc Natl Acad Sci USA.* 1989; 90:9193–9197.

13. Plowman GD, Culouscou JM, Whitney GS. Ligand-specific activation of HER-4/p180erbB4, a fourth member of the epidermal growth factor family. *Proc Natl Acad Sci USA.* 1993;90:1746–1750.

14. Hancock MC, Langton BC, Chan T, et al. A monoclonal antibody against the c-erbB-2 protein enhances the cytotoxicity of cis-diaminedichloroplatinum against human breast and ovarian tumor cell lines. *Cancer Res.* 1991;51:4575–4580.

15. Stancovski I, Hurwitz E, Leitner D, et al. Mechanistic aspects of the opposing effects of monoclonal antibodies to the erB-2 receptor on tumor growth. *Proc Natl Acad Sci USA.* 1991;88:8691–8695.

16. Carter P, Presta L, Gorman CM, et al. Humanization of an anti-p185HER2 antibody

for human cancer therapy. *Proc Natl Acad Sci USA*. 1992;89:4285–4289.

17. Coussens L, Yang-Feng TL, Liao Y-C, et al. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. *Science*. 1985;230:1132–1139.

18. Yokota J, Yamamoto N, Miyajima K, et al. Genetic alterations of the c-erbB-2 oncogene occur frequently in tubular adenocarcinoma of the stomach and are often accompanied by amplification of the v-erbA homologue. *Oncogene*. 1988;2:283–287.

19. Hudziak RM, Lewis GD, Winget M, et al. P185HER2 monoclonal antibody has antiproliferative effects in vivo and sensitizes human breast tumor cells to tumor necrosis factor. *Mol Cell Biol*. 1989;9: 1165–1172.

20. Kraus MH, Popescu NC, Amsbaugh C, et al. Overexpression of the EGF receptor–related proto-oncogene erbB-2 in human mammary tumor cell lines by different molecular mechanisms. *EMBO J*. 1987;6:605–610.

21. Haigler H, Ash JF, Singer J, et al. Visualization by fluorescence of the binding and internalization of epidermal growth factor in human carcinoma cells. *Proc Natl Acad Sci USA*. 1978;75:3317–3321.

22. Lewis GD, Figari I, Fendly B, et al. Differential responses of human tumor cell lines to anti-p185 HER2 monoclonal antibodies. *Cancer Immunol Immunother*. 1993; 37:255–263.

23. Pietras RJ, Fendly BM, Chazin VR, et al. Antibody to HER-2/neu receptor blocks DNA repair after cisplatin in human breast and ovarian cancer cells. *Oncogene*. 1994;9:1829–1838.

24. Baselga J, Norton L, Albanell J, et al. Recombinant humanized anti-HER2 antibody (herceptin) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/neu overexpressing human breast cancer xenografts. *Cancer Res*. 1998;58:2825–2831.

25. Data on file. Genentech, Inc. HHS U.S. license #1048;1998.

26. Baselga J, Tripathy D, Mendelsohn J, et al. Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. *J Clin Oncol*. 1996;14:737–744.

27. Cobleigh M. Efficacy and safety of herceptin (humanized anti-HER-2 antibody) as a single agent in 222 women with HER-2 overexpression who relapsed following chemotherapy for metastatic breast cancer. In: Program and abstracts of the American Society of Clinical Oncology meeting. 1998;17:376. Abstract.

28. Slamon DJ, Leyland-Jones B, Shak S, et al. Addition of herceptin (humanized anti-HER-2 antibody) to first line chemotherapy for HER-2 overexpressing metastatic breast cancer markedly increases anti-cancer activity: In: Program and abstracts of the American Society of Clinical Oncology meeting. 1998;17:377. Abstract.

MINIREVIEW

Challenges in the Development of High Protein Concentration Formulations

STEVEN J. SHIRE,[1] ZAHRA SHAHROKH,[2] JUN LIU[1]

[1]Department of Pharmaceutical R&D, Genentech, Inc., South San Francisco, California 94080

[2]Department of Pharmaceutical and Analytical Development, Transkaryotic Therapies, Cambridge, Massachusetts 02139

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ABSTRACT: Development of formulations for protein drugs requiring high dosing (in the order of mg/kg) may become challenging for solubility limited proteins and for the subcutaneous (SC) route with <1.5 mL allowable administration volume that requires >100 mg/mL protein concentrations. Development of high protein concentration formulations also results in several manufacturing, stability, analytical, and delivery challenges. The high concentrations achieved by small scale approaches used in pre-formulation studies would have to be confirmed with manufacturing scale processes and with representative materials because of the lability of protein conformation and the propensity to interact with surfaces and solutes which render protein solubilities that are dependent on the process of concentrating. The concentration dependent degradation route of aggregation is the greatest challenge to developing protein formulations at these higher concentrations. In addition to the potential for nonnative protein aggregation and particulate formation, reversible self-association may occur, which contributes to properties such as viscosity that complicates delivery by injection. Higher viscosity also complicates manufacturing of high protein concentrations by filtration approaches. Chromatographic and electrophoretic assays may not accurately determine the non-covalent higher molecular weight forms because of the dilutions that are usually encountered with these techniques. Hence, techniques must be used that allow for direct measurement in the formulation without substantial dilution of the protein. These challenges are summarized in this review. © 2004 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 93:1390–1402, 2004

Keywords: protein formulation; high concentration; pharmaceutical; manufacturing; analytical; viscosity

INTRODUCTION

Over the past two decades, recombinant DNA technology has led to the commercialization of many protein therapeutics requiring successful formulations. The most conventional route of delivery for protein drugs has been intravenous (IV) administration because of poor bioavailability by most other routes, greater control during clinical administration, and faster pharmaceutical development. For products that require frequent and chronic administration, the alternate subcutaneous (SC) route of delivery is more appealing. Particularly when coupled with prefilled syringe and autoinjector device technology, SC delivery allows for home administration and improved compliance of administration, and may

Correspondence to: Steven J. Shire (Telephone: 650-225-2077; Fax: 650-225-7234; E-mail: steve_shire@gene.com)

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result in expanded product markets. Proteins such as monoclonal antibodies are often administered with frequent dosing regimens and at high doses (several mg/kg). Two antibodies, Rituxan® and Herceptin® that have been approved for the treatment of cancer are intravenously administered in hospitals, but several programs are underway for use of monoclonal antibodies to treat diseases that may require outpatient administration, and hence require the development of SC route of administration. Treatments with high doses, e.g., more than 1 mg/kg or 100 mg per dose, require development of formulations at concentrations exceeding 100 mg/mL because of the small volume (<1.5 mL) that can be given by the SC routes. For some proteins, that have limited solubility, achieving such high concentration formulations may require the use of solubility enhancers. Even for the IV delivery route, where large volumes can be administered, protein concentrations of tens of milligram per milliliter may be needed for high dosing regimens and this may pose solubility challenges for some proteins such as cytokines and enzymes. Development of formulations at high concentrations also poses stability, manufacturing, and delivery challenges related to the propensity of proteins to aggregate at the higher concentrations. These challenges and appropriate analytical techniques for assessing high concentration protein formulations will be discussed.

SOLUBILITY CONSIDERATIONS

A potential challenge to developing a protein formulation that enables the desired dosing is achieving the required protein concentration in solution. The desired protein concentration may be difficult to achieve for some enzymes and cytokines that have limited solubility. The principles governing protein solubility are more complicated than those for small synthetic molecules,[1,2] and thus overcoming the protein solubility issue takes different strategies. Operationally, solubility for proteins could be described by the maximum amount of protein in the presence of co-solutes whereby the solution remains visibly clear (i.e., does not show protein precipitates, crystals, or gels,), or does not sediment at 30,000*g* centrifugation for 30 min.[3] The dependence of protein solubility on ionic strength, salt form, pH, temperature, and certain excipients has been mechanistically explained by changes in bulk water surface tension and protein binding to water and ions versus self-association.[1,3–5] Binding of proteins to specific excipients or salts influences solubility through changes in protein conformation or masking of certain amino acids involved in self-interaction. Proteins are also preferentially hydrated (and stabilized as more compact conformations) by certain salts, amino acids, and sugars, leading to their altered solubility.[5] Protein solubility could also depend on the level of purity of the protein preparation; e.g., fibrinogen solubility was found to be dependent on the initial amount of protein because of the impurities and heterogeneity of the preparation.[6] Hence, selection of a protein formulation based on preformulation work that uses materials from early process development runs may be a misguided decision and needs to be verified with representative larger scale preparations.

Determining the highest protein concentration achievable remains an empirical exercise because the lability of protein conformation and the propensity to interact with itself, with surfaces, and with specific solutes, all lead to protein solubilities that depend on the process of concentrating. Table 1 lists the various methods for concentrating a protein solution, with their advantages and practical limitations. Early in preformulation studies where material is limited, small-scale methods are needed to achieve high protein concentrations. The most mechanically gentle and rapid methods involve osmotic pressure dependent microdialysis principles.[7,8] One such method[7] very rapidly achieves several orders of magnitude increase in protein concentration simultaneous with buffer exchange using only ~0.1 mg protein. Though this approach is a useful tool for rank ordering of solubility in various formulation compositions, for some cytokines, it gave 10-times higher solubilities than those reached by dialfiltration/ultrafiltration on a Centricon® system that may result in shear induced protein denaturation and lowered apparent solubility (Shahrokh et al., unpublished results). Another approach based on osmotically driven solute concentration involves dialysis against a hygroscopic material such as Sephadex or polyethylene glycols.[2] This approach requires the use of high purity materials that do not result in protein degradation, and must be done with care to prevent over drying and exposure to very large surface areas since water rapidly leaves the protein solution. In addition, as polyelectrolytes, proteins impact the distribution of diffusible ions across the dialysis membrane to maintain

Table 1. The Commonly Used Methods of Concentrating Proteins

Concentrating Method	Scale	Benefits	Limitations	Used for Solubility Determination
Osmotic driven (dialysis) methods	Few μL to few mL	Small-scale; mechanically gentle; rapid	Concentrations achieved may not translate to manufacturing scale	Yes
Against solutions		Simultaneous buffer exchange		
Against water absorbing materials			Have to buffer exchange later	
Solvent evaporation				
Vacuum, N2 flow	Few μL to few mL	Small-scale	Slow (may lead to degradation); concentrates excipients	Yes, only in solution composition reached at end of process
Lyophilization	mL	Pharmaceutically relevant and scalable	Must protect against drying induced degradation; concentrates excipients	
Precipitation (salt or solvent; SCF)	Few μL to L	Quick and scalable	Solution composition not pharmaceutically relevant May lead to irreversible degradation	No
Freezing	Few mL to L	Tens of fold concentration is achieved quickly	Concentrates excipients too Process may damage protein	No
Chromatographic bind and elute	Few μL to many mL	Wide range of scale	Solution composition not pharmaceutically relevant Conditions may damage protein	No
Filtration Systems				Yes, if not material limited
Centrifugal (Centricon)	Hundreds of μL to tens of mL	Moderate scale; may include buffer exchange	Adsorption losses; shear induced degradation Findings may not translate to manufacturing scale	
Pressure (Amicon)	Hundreds of mL to L	Manufacturing scale; may include buffer exchange	Large scale impractical for early development phases	
TFF			High viscosity or pore clogging may limit concentrations reached	

electroneutrality as dictated by the Donnan equilibrium.[9] This could lead to a potentially large change in the ion composition within the protein solution as it concentrates, and could be damaging to the protein. The alternative small-scale methods of solvent evaporation by vacuum or nitrogen flow, and precipitation by salts or solvents,[10–12] alter the formulation composition and may also result in protein degradation due to the time or materials used in the process. Precipitation and generation of protein powders by supercritical fluids has met some success in maintaining active or structurally native proteins, but this approach remains of limited practical use particularly for small scale studies.[13,14] Concentration of solutes by freezing[15] is another approach that could achieve a high protein concentration, but alteration in the solute composition, potential damage to the protein during freezing and thawing, and difficulty in retrieving the frozen concentrate all make this an impractical approach. Chromatographic methods that bind the protein and allow elution at lower volumes is another useful tool for concentrating proteins over a large range of scale; these include ion exchange or hydrophobic interaction chromatography, but require buffer exchange after concentration. Filtration is the most common approach for concentrating proteins, and unlike many of the methods discussed is amenable to scale-up for manufacturing. There are numerous filtration systems, each with an extent of adsorption or shear-induced denaturation/aggregation that is dependent on surface area, contact materials, and flow mechanisms. The mechanical and interfacial forces imposed on protein's structure (hence its solubility) by small-scale methods may be different than those by the large-scale processes, and hence, the results obtained by such methods would have to be verified by representative manufacturing processes. The most commonly used methods for large scale manufacturing of high protein concentrations are further discussed in "Manufacturing Considerations."

STABILITY CONSIDERATIONS

Although proteins are more complex than typical small molecule pharmaceuticals, a great deal is now known about the various chemical degradations that occur in proteins, and several reviews are available.[16–18] Many of the chemical reactions such as deamidation, aspartate isomerization, oxidation, and peptide bond hydrolysis that occur in proteins are hydrolytically driven requiring the presence of water, and generally the kinetics follows lower order concentration dependency. Aggregation, on the other hand, requiring bi-molecular collisions, and a high concentration dependency is expected to be the primary degradation pathway in high concentration protein formulations. The relationship of concentration to aggregate formation depends on the size of aggregates as well as the mechanism of association.[12,17] Protein aggregation may result in covalent (e.g., disulfide-linked) or non-covalent (reversible or irreversible) association. Irreversible aggregation by non-covalent association generally occurs via hydrophobic regions exposed by thermal, mechanical, or chemical processes that alter a protein's native conformation.[19] When the protein-unfolding step is rate limiting, the kinetics of protein aggregation often shows pseudo-first order behavior[19] rather than the expected higher order concentration dependency. Further illustration of the importance of the aggregation mechanism on the relationship of concentration to aggregate formation is shown by the inverse concentration dependency of protein aggregation that results from interaction with air–water interfaces.[20]

Protein aggregation may impact protein activity, pharmacokinetics and safety, e.g., due to immunogenicity.[18,21,22] Irreversible protein aggregation is recognized as a major degradation product in protein formulations, whereas reversible protein self-association is often overlooked. Reversible protein association is less studied, partly because of poor analytical methodologies (as will be discussed under "Analytical Considerations") and partly because of the perception that aggregates will not be present after typical dilutions are made prior to administration. However, if the rate of dissociation is slow, then reversible aggregation may still impact activity, *in vivo* clearance and safety. Even under conditions where the rate of dissociation is rapid, the equilibrium at high protein concentration may be shifted toward a greater amount of aggregate due to molecular crowding effects.[23–25] In particular, as the protein concentration increases, the fraction of the total volume occupied by the protein increases. The resulting decrease in the effective volume available to the protein yields a higher apparent protein concentration that in turn favors self-association. This non-ideal solution behavior of increasing the apparent thermodynamic association constant with increasing protein concentration may be shelf-life limiting, especially when the resulting

aggregates are irreversible. In addition, after *in vivo* administration, the protein therapeutic may be placed into a crowded macromolecular environment, which shifts the equilibrium to the associated state even after dilution.

Macromolecular crowding, i.e., excluded volume effects, also can impact protein physical properties such as viscosity which can have major impact on the ability to manufacture high protein concentration formulations (see "Manufacturing Considerations") as well as on the ability to administer the protein drug by injection (Fig. 2A). In general, the viscosity of a macromolecule in solution can be expressed as a virial expansion, where the viscosity, η, can be related to the solvent viscosity, η_0, and concentration of the protein, c_p, in g/mL by a power series[26]:

$$\eta = \eta_o\left(1 + k_1c_p + k_2c_p^2 + k_3c_p^3 + \cdots\right) \quad (1)$$

where in equation 1, k_1 is the intrinsic viscosity, which is related to the contribution from individual solute molecules, and k_2 and higher order coefficients are related to effects from interactions of 2, 3, or more protein molecules. This equation can be rewritten in terms of the specific viscosity, $\eta_s = (\eta - \eta_o)/\eta_o$:

$$((\eta - \eta_o)/\eta_o)/c_p = \eta_s/c_p = k_1 + k_2c_p + k_3c_p^2 + \cdots \quad (2)$$

In the case of significant protein self-association that results in formation of soluble aggregates, the higher order terms will dominate and lead to large increases in viscosity as a function of concentration.

There are two key approaches to mitigating irreversible protein aggregation for improving the shelf life of high concentration protein formulations. One approach uses conditions that stabilize the native conformation of the protein, and the other, reduces molecular collisions that lead to aggregation. For the first approach, molar concentrations of osmolytes, such as sugars, are added that are preferentially excluded from the immediate environment of proteins, and result in preferential hydration.[27,28] This preferential hydration leads to an increase in the protein's chemical potential since the increased ordering of water in the hydration shell leads to an entropic decrease for the entire thermodynamic system consisting of protein and solvent water. Since a larger surface area such as that found in unfolded proteins requires a greater number of ordered water molecules for preferential hydration, the protein will assume a more compact folded form to resist the thermodynamically unfavorable decrease in the system's entropy. Thus, the protein will minimize the surface area available for hydration leading to stabilization of the more compact folded form, which then decreases the formation of protein aggregates. However, if the native state of the protein is capable of associating, forming aggregates of native conformers, then the addition of preferentially excluded osmolytes may actually increase the amount of protein aggregate since total surface area will be diminished following protein self association. The potential impact of reversible self-association on product shelf-life or protein physical parameters will depend on the rate of dissociation as well as the possible consequent formation of irreversible aggregates during storage at high concentration. The use of preferentially excluded osmolytes to fix the aggregation problem is limited since addition of high concentrations of excipients may also add to the viscosity and osmolality of the formulation that may render it impractical for use as a parenteral solution. Also, as already noted, the addition of these osmolytes may actually increase the amount of reversible native associated protein.

The second approach to minimize aggregation is to restrict the mobility of proteins in order to reduce the number of collisions. Lyophilization with appropriate excipients may improve protein stability against aggregation by decreasing protein mobility and by restricting conformational flexibility with the added benefit of minimizing hydrolytic reactions consequent to removal of water.[29] The addition of appropriate excipients, including lyoprotectants, prevents the formation of aggregates during the lyophilization process as well as during storage of the final product.[30–32] A key parameter for effective protection is the molar ratio of the lyoprotectant to the protein.[33] Generally molar ratios of 300:1 or greater are required to provide suitable stability, especially for room temperature storage. While this strategy achieves an optimal final formulation at moderate protein concentrations, if the lyophilizer is used to concentrate the protein, additional challenges can result as discussed below.

MANUFACTURING CONSIDERATIONS

Although it may be possible to create a formulation during development that has the desired solubility and stability, the design of processes

that will allow for manufacturing of the formulation at a larger scale is critical for successful implementation of bench top experimental findings. Two commonly used processes will be discussed below.

Tangential Flow Filtration (TFF)

The main technology for buffer exchange and concentrating proteins that has been used at commercial scale is TFF.[34–36] In this technology, the protein is circulated by pumping through a series of hollow fiber tubes, which allow for the passage of water and small molecules but not large macromolecules. Attainment of high concentration formulations by TFF systems can be difficult because the required membrane flux may dictate higher concentrations at the membrane boundary than the targeted concentration. As an example, when a protein is concentrated to 100 mg/mL, the concentration at the membrane may be as high as 125 mg/mL. Depending on a protein's propensity to interact with and unfold at the surface, this could lead to decreased flux and eventual membrane clogging. Proteins are often shear sensitive, resulting in denaturation,[37,38] and the continuous circulation through tubings and pulsation of the pumps may generate sufficient shear or cavitation to result in protein unfolding and precipitation. Although it may be possible to filter out the precipitated protein during manufacture of bulk protein, the shear sensitivity of a protein may also lead to formation of aggregates and particulates during filling of the final product that requires some sort of a pumping system. Typical filling systems include piston driven and diaphragm pumps. Piston driven pumps tend to generate more shear than rolling diaphragm pumps and this needs to be considered when filling high concentration protein solutions.

Another parameter that could limit a TFF system's ability to concentrate a protein solution is the solution's high viscosity. The viscosity increase may result in such high back-pressures during the TFF process that it may exceed the capacity of the pumps. In addition, as viscosity increases, it becomes increasingly difficult to remove the final finished product from the TFF unit, leading to economically unacceptable losses. Though these limitations may be handled by appropriate TFF equipment redesign, equipment design can be an expensive proposition. Since the viscosity of a macromolecular solution is dependent on temperature, increased temperatures would improve the TFF process if the protein stability is not compromised. Alternatively, design of an appropriate formulation to decrease viscosity may help solve manufacturing process limitations. Hence, the formulation may need to be designed to ensure not only acceptable shelf life, but also compatibility with the manufacturing process.

Although TFF is the industry standard for concentrating macromolecules at large scale, there are alternatives. Rotating and vortex systems that enhance mass-transfer have recently been designed.[36,39] Alternatively, any drying technique with a subsequent reconstitution at lower volumes can be used as will be discussed below.

Drying Techniques for Generating High Concentration Protein Formulations

As discussed earlier in "Solubility Considerations," a high concentration protein formulation can be achieved either by decreasing the solvent content while retaining the protein, e.g., using filtration systems, or by removal of solvent followed by reconstitution with the desired solvent volume. The latter can be achieved either by bulk processing or by dehydrating final product in vials. Bulk drying processes include spray drying,[40,41] fluid bed drying,[42,43] vacuum drying,[44,45] or lyophilization in tray units.[46] Crystallization has proven to be effective in the development of insulin formulations, and shows great promise for the development of stable monoclonal antibody dosage forms.[47,48] For parenteral protein products, bulk drying processes require operation under aseptic conditions, and have been used primarily in the aerosol industry. The alternative approach of lyophilizing sterile filtered products in vials is a cost-effective approach for generating high concentration protein formulations.

To use the lyophilizer as a concentrator, a loading volume of protein, V_L, at loading concentration C_L is lyophilized and then reconstituted with a volume of diluent, V_R, where $V_R < V_L$. The final concentration of the drug product, C_F, will then be:

$$C_F = C_L V_L / (V_R + V_S) \quad (3)$$

where V_S is the volume contributed from the remaining solids. To determine the appropriate V_R that gives a desired C_F, one can estimate V_S from the sum of the partial molar volumes of all the excipients. However, the most accurate way to achieve the target protein concentration is to reconstitute with a series of diluent volumes and empirically determine the appropriate reconstitution

volume. In addition to reaching the desired concentration, sufficient volume needs to be used in the reconstitution process to ensure that the required volume for administration can be removed from the vial. Thus, experiments may need to be designed whereby V_L and/or C_L are also varied to define the required overrage in the final reconstituted product. These parameters can also have a major impact on the reconstitution properties of the final product. Figure 1 shows the effect of loading concentration on reconstitution times and morphology of the lyophilized solid. Each vial was loaded with the same total mass of protein and formulation excipients (i.e., a lower C_L required a larger V_L to maintain the loading mass). Vials were reconstituted to a final concentration of 125 mg/mL and investigated for solution clarity and disappearance of all solids after 10 min. The right side of Figure 1 shows that as the protein loading concentration is decreased, reconstitution occurs much more readily. Investigation of the morphology of the lyophilized solid by scanning electron microscopy (SEM; left side of Fig. 1) shows a very dense compact structure at 110 mg/mL compared to a loosely packed layer of flakes at 40 mg/mL. Thus, the ease of wetting of the cake is very likely related to the differences in this morphology. SEM analysis of intermediate concentrations (not shown) demonstrates a gradual transition from a dense solid to the more loosely packed structure.

The process described above allows for designing a formulation with appropriate stability and tonicity. Although isotonicity is not necessarily required for SC administration, it may be desirable for minimizing pain upon administration.[49] Isotonicity is difficult to achieve because both the protein and the excipients are concentrated during the reconstitution process. As shown in Table 2, excipient:protein molar ratios of 500:1 will result in hypertonic preparations if the final protein concentration is targeted for >100 mg/mL. If the desire is to achieve an isotonic formulation, then a choice of lower molar ratio of excipient:protein will result in a potentially less stable formulation. This was indeed the issue involving a rhuMAb preparation where the hypertonic formulation at 500:1 excipient:protein molar ratio gave a significantly

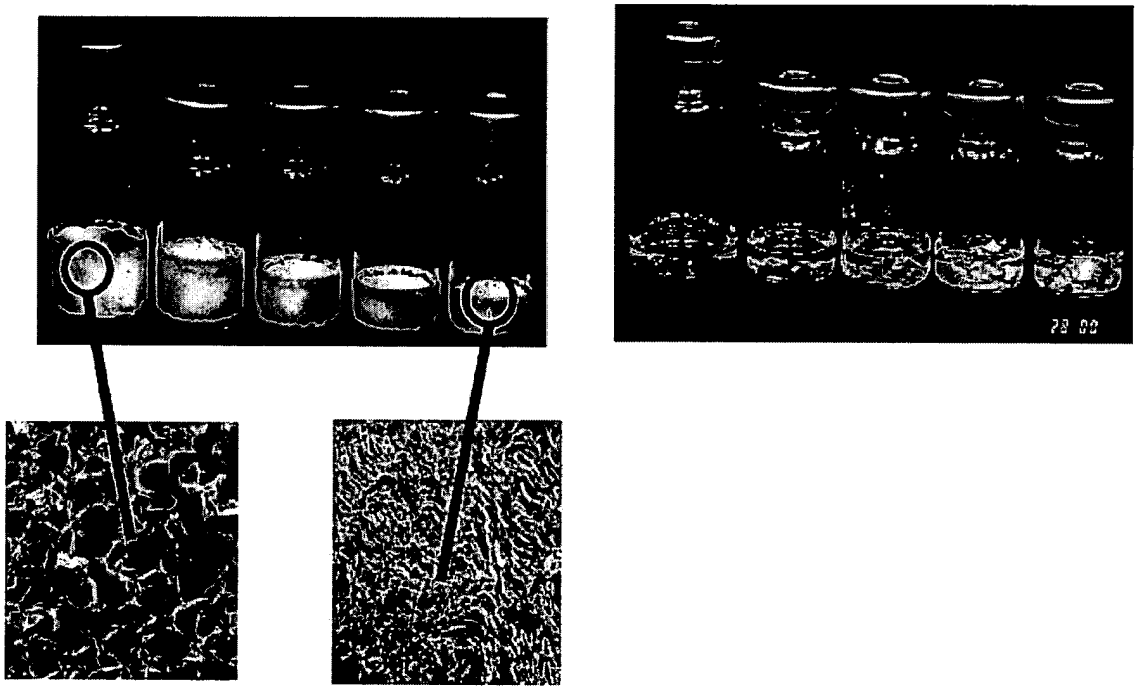

Figure 1. Lyophilization of a monoclonal antibody as a function of loading concentration. Upper left panel: Loading concentration from left to right was 40, 60, 80, 100, and 110 mg/mL, respectively, while maintaining the same total mass of MAb and excipients. Lower left panels: Scanning electron microscopy of lyophilized solid for the 40 and 110 mg/mL MAb loading concentrations. Right panel: 10 min after reconstitution of vials in upper left panel to 125 mg/mL with sterile water for injection.

Table 2. Concentration of Excipient as a Function of Final Reconstituted Protein Concentration and Molar Ratio of Excipient to Protein

Excipient:Protein Molar Ratio	250:1	500:1
C_F (mg/mL)	Excipient concentration (mM)	Excipient concentration (mM)
50	83	167
100	167	333
150	250	500
200	333	666

more stable preparation at controlled room temperature (30°C), but the stabilities at 250 and 500:1 molar ratios were comparable at 2–8°C storage (data not shown). In the case of this particular antibody where the target product profile and frequency of administration allowed for 2–8°C storage, stability was traded off for appropriate tonicity, and the 250:1 molar ratio was selected.

COST OF GOODS AND DELIVERY CONSIDERATIONS

Though high concentration formulations have the cost saving advantage of decreasing bulk storage space or number of product fills, they have undesirable overall cost of goods because of unrecoverable volumes from ultrafiltration units, fill vessels, and product containers. As an example, whereas the required volume overrage in vials containing moderate protein concentrations is typically <10% of the fill volume, the overrage for a >100 mg/mL protein formulation could be as high as 30% because of greater adherence of the viscous solution to product container surfaces. Unrecoverable product losses in the final dosage form could be minimized by decreasing product contact surface areas, e.g., with the use of smaller or narrower based vials, or by using prefilled syringe configurations where the solution can be pushed off the surfaces with the plunger head.

Protein formulations at high concentrations may also have physical properties that impact the ability to easily deliver the protein drug. For example, higher viscosity preparations may be difficult to administer by injection. Syringes for SC injection are often equipped with 26 or 27 gauge needles. If the viscosity of a high concentration formulation is sufficiently high, it may impact the ability to load and deliver from a syringe, and unless the viscosity can be reduced by appropriate formulation excipients, the high concentration required for SC delivery may not be attainable. As an example, the protein concentration dependence of viscosity of an antibody is shown in Figure 2A. The time required to load 1 mL of this formulation into a syringe equipped with 27 gauge needle correlates very well with the viscosity of the formulation. Adjustment of the formulation to contain NaCl greatly reduces viscosity (Fig. 2B),

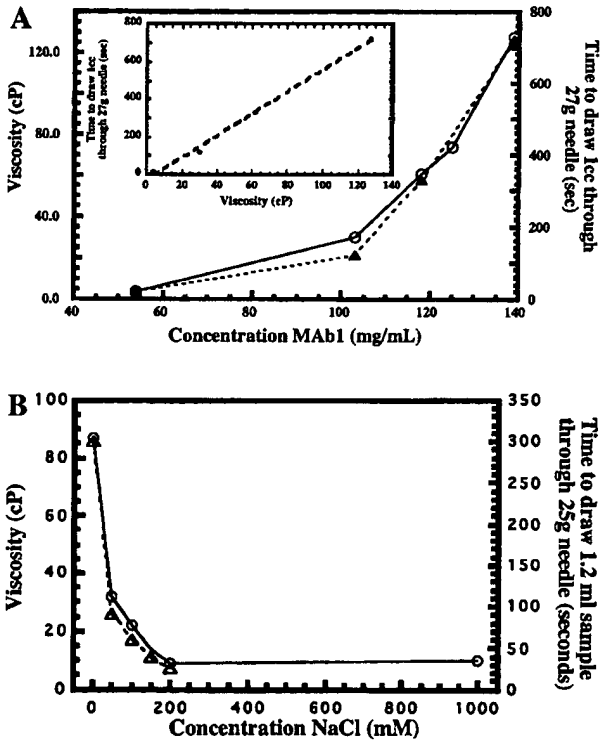

Figure 2. A: Viscosity (solid line, open circles) and syringe (27 gauge) loading time (dashed line, solid triangles) of a monoclonal antibody as a function of concentration. Insert is a linear regression fit of syringe (27 gauge) loading time versus viscosity (loading time = $-25.9 + 5.8$*viscosity, $R^2 = 0.995$. B: Viscosity (solid line, open circles) and syringe (25 gauge) loading time of a monoclonal antibody at 125 mg/mL as a function of NaCl concentration.

and yields reasonable times to draw the formulation into a syringe equipped with a 25 gauge needle. Thus, the alteration of the formulation excipients and the use of a slightly bigger needle yield reasonable times for withdrawal of a high concentration MAb formulation.

ANALYTICAL CONSIDERATIONS

Many of the analytical techniques currently available to explore covalent and conformational modifications in proteins[50,51] are easily adapted to the study of proteins at high concentration. Moreover, analytical techniques that characterize solid-state protein dosage forms are useful in developing drying methods to produce a high protein concentration subsequent to reconstitution. These techniques include differential scanning calorimetry (DSC),[52] particularly modulated DSC technology,[53,54] to determine glass transition temperatures and thermal phase transitions of the solid-state matrix at high protein concentrations, Fourier-Transform Infrared Spectroscopy (FTIR)[55,56] and Raman spectroscopy to study the protein's secondary structure after drying and during storage, and fluorescence spectroscopy recently used to investigate possible tertiary structural alterations during the drying process.[57]

Generally the analytical technologies that are used to characterize proteins involve dilution to lower concentrations or exposure of the protein to solvent conditions that are very different than the initial formulation composition. This may have considerable impact on the results of the assay since a change in solvent composition or concentration may alter a protein's physical state in a way that is not representative of the initial conditions. This problem is especially important in the analysis of molecular weight/size distribution of proteins. SDS–PAGE, nongel sieving SDS-capillary electrophoresis, and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) are often used to obtain molecular weight information. These techniques are useful for detecting covalently linked aggregates, or SDS non-dissociable aggregates but can't be used to determine non-covalent protein association states. The often-used gel permeation or size exclusion chromatographic method (SEC) provides such information but has several problems. The protein's interaction with the chromatographic resin and its hydrodynamic volume may alter the elution times; the use of globular protein standards to estimate the molecular weight may thus be erroneous. When the interactions are ionic in nature due to the charge moieties on the resins, the addition of salts will decrease such interactions, but control experiments are then required to demonstrate that the increased ionic strength of the mobile phase did not perturb the size distribution of the protein. The impact of hydrodynamic volume leading to apparently larger proteins than actual size is especially noted for highly glycosylated proteins that have shapes different from the typical globular protein standards.[58,59] The use of static light scattering detectors coupled with sizing chromatography (LC/LS)[60] allows for the absolute determination of molecular mass of a protein and its higher order aggregates and fragments during separation by gel sieving. Since measured elution times are no longer used to estimate molecular size, the problem of protein-resin interaction and molecular conformation are no longer important, provided the interactions do not prevent elution of the protein from the chromatographic column.

The most commonly used analytical technique for quantifying aggregates in pharmaceutical formulations is SEC with UV detection. In high concentration formulations where reversible protein self-association predominates, the determination of aggregate levels by the SEC method may be inconsistent and inaccurate. The SEC procedure involves a high dilution of the protein solution during injection onto the column that may lead to dissociation of aggregates with rapid dissociation rate constants. Moreover, to prevent detector saturation, high concentration protein solutions are generally diluted even prior to injection onto the SEC column. The rapid dissociation of a monoclonal antibody upon dilution[61] yielded varying aggregate levels by SEC, depending on the time and temperature of analysis after sample dilution. To use SEC as an analytical tool for quantification of aggregates and estimation of formulation's shelf life and physical characteristics, it is imperative to have reproducible measurements. For a slower dissociating protein in the example shown in Figure 3, reproducible quantification of aggregates in an 80 mg/mL formulation is achieved by limiting the analysis time to less than 24 h and by controlling the most critical parameters, the protein concentration after dilution and sample temperature (a 5-fold decrease in dissociation rate was observed for every 10°C decrease in temperature of the auto-sampler compartment of the HPLC). The clinically relevant aggregate level for

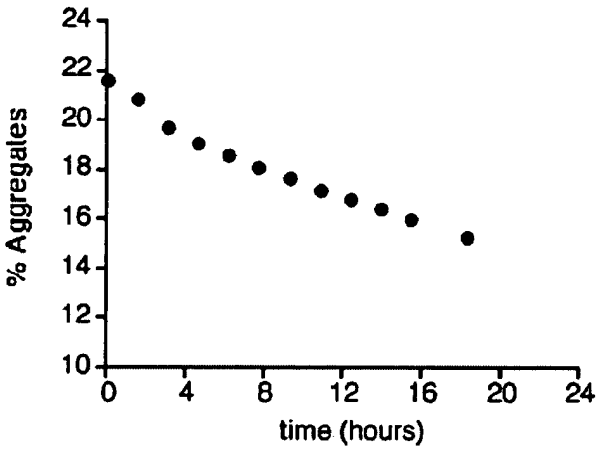

Figure 3. Dissociation of aggregates in an 80 mg/mL protein formulation at 30°C upon dilution to 0.1 mg/mL. At 5°C, the dissociation rate is too small to detect any changes in the initial aggregate level during this time frame.

a protein that undergoes reversible self-association is that which remains after dilution in the IV solution for administration (if applicable) and after initial dilution in blood volume at 37°C. This may have to be determined separately from the routine stability testing of the pharmaceutical product, particularly when dissociation rate is slower than practical for routine analyses.

Technologies such as static and dynamic light scattering[62–65] and analytical ultracentrifugation[62–65] provide more representative information about protein self-association states at high protein concentrations because they maintain the concentration during analysis and can also be performed at high concentration. Another technique uses preparative centrifuges coupled with a specially designed microfractionator to obtain sedimentation equilibrium measurements of molecular weight in concentrated as well as multicomponent protein systems.[66] Limitations of the light scattering methods are that they are not quantitative and that they are subject to multiple scattering artifacts at high protein concentrations. Although sedimentation equilibrium centrifugal analysis can be used to characterize an aggregating system, the analysis requires fitting of data to several exponentials and is model dependent.[67] Sedimentation velocity analysis has been greatly improved and algorithms are now available to determine small amounts of protein aggregates and sedimentation coefficients.[68,69] The determination of the sedimentation coefficient can provide valuable information regarding overall shape of the protein when coupled with computations of hydrodynamic bead models.[70,71] However, correction of apparent molecular weights and sedimentation coefficients due to nonideality at high protein concentrations is difficult to achieve.

SUMMARY AND CONCLUSIONS

Protein properties such as self-association/aggregation, solubility, and viscosity pose challenges to developing pharmaceutically and economically acceptable formulations at high concentration. In addition to maintaining suitable stability, protein properties at high concentration may impact the ability to administer the drug, to manufacture at large scale, and also the yields of the two processes. Analytical methodologies to investigate protein properties at high concentration are also limited and must be considered with caution as they are impacted by the very property under investigation. Very little work has been published on high concentration protein formulation development and this review has touched on the key issues with examples of the potential solutions to the issues. Achieving a suitable formulation requires an integrated approach whereby a stable formulation is developed that can also be successfully administered and economically manufactured.

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REFERENCES

1. Arakawa T, Timasheff SN. 1985. Theory of protein solubility. Methods Enzymol 114:49–77.
2. Middaugh CR, Volkin DB. 1992. Protein solubility. In: Ahern TJ, Manning MC, editors. Stability of protein pharmaceuticals, 1st edn., New York: Plenum Press. p 109–134.
3. Schein CH. 1990. Solubility as a function of protein structure and solvent components. BioTechnol 8(4):308–317.
4. Melander W, Horvath C. 1977. Salt effect on hydrophobic interactions in precipitation and chromatography of proteins: An interpretation of the

lyotropic series. Arch Biochem Biophys 183(1):200–215.
5. Jenkins WT. 1998. Three solutions of the protein solubility problem. Protein Sci 7(2):376–382.
6. Leavis PC, Rothstein F. 1974. The solubility of fibrinogen in dilute salt solutions. Arch Biochem Biophys 161(2):671–682.
7. Zhang R, Hjerten S. 1997. A micromethod for concentration and desalting utilizing a hollow fiber with special reference to capillary electrophoresis. Anal Chem 69(8):1585–1592.
8. Saul A, Don M. 1984. A rapid method of concentrating proteins in small volumes with high recovery using Sephadex G-25. Anal Biochem 138(2):451–453.
9. Bull HB. 1971. An introduction to physical biochemistry, 2nd edn., Philadelphia: F.A. David Company. p 174.
10. Rothstein F. 1994. Differential precipitation of proteins. Science and technology. Bioprocess Technol 18:115–208.
11. Tanabe S, Tesaki S, Watanabe M. 2000. Producing a low ovomucoid egg white preparation by precipitation with aqueous ethanol. Biosc Biotechnol Biochem 64(9):2005–2007.
12. Glatz CE. 1992. Modeling of aggregation-precipitation phenomena. In: Ahern TJ, Manning MC, editors. Stability of protein pharmaceuticals, 1st edn., New York: Plenum Press. p 135–166.
13. Moshashaee S, Bisrat M, Forbes RT, Quinn EA, Nyqvist H, York P. 2003. Supercritical fluid processing of proteins: Lysozyme precipitation from aqueous solution. J Pharm Pharmacol 55(2): 185–192.
14. Winters MA, Knutson BL, Debenedetti PG, Sparks HG, Przybycien TM, Stevenson CL, Prestrelski SJ. 1996. Precipitation of proteins in supercritical carbon dioxide. J Pharm Sci 85(6):586–594.
15. Webb SD, Webb JN, Hughes TG, Sesin DF, Kincaid AC. 2002. Freezing bulk-scale biopharmaceuticals using common techniques—And the magnitude of freeze-concentration. Biopharm 15(5):2–8.
16. Ahern TJ, Manning MC, editors. 1992. Stability of protein pharmaceuticals. Part A: Chemical and physical pathways of protein degradation, 1st edn., New York: Plenum Press. p 434.
17. Manning MC, Patel K, Borchardt RT. 1989. Stability of protein pharmaceuticals. Pharm Res 6(11):903–918.
18. Cleland JL, Powell MF, Shire SJ. 1993. The development of stable protein formulations: A close look at protein aggregation, deamidation and oxidation. Crit Rev Ther Drug Carrier Syst 10(4):307–377.
19. Chi EY, Krishnan S, Randolph TW, Carpenter JF. 2003. Physical stability of proteins in aqueous solution: Mechanism and driving forces in nonnative protein aggregation. Pharm Res 20(9):1325–1336.
20. Treuheit MJ, Kosky AA, Brems DN. 2002. Inverse relationship of protein concentration and aggregation. Pharm Res 19(4):511–516.
21. Braun A, Kwee L, Labow MA, Alsenz J. 1997. Protein aggregates seem to play a key role among the parameters influencing the antigenicity of interferon alpha (IFN-alpha) in normal and transgenic mice. Pharm Res 14(10):1472–1478.
22. Koren E, Zuckerman LA, Mire-Sluis AR. 2002. Immune responses to therapeutic proteins in humans—Clinical significance, assessment and prediction. Curr Pharm Biotechnol 3(4):349–360.
23. Minton AP. 1992. Confinement as a determinant of macromolecular structure and reactivity. Biophysical J 63(4):1090–1100.
24. Wilf J, Minton AP. 1981. Evidence for protein self-association induced by excluded volume. Myoglobin in the presence of globular proteins. Biochimica et Biophysica Acta 670(3):316–322.
25. Zimmerman SB, Minton AP. 1993. Macromolecular crowding: Biochemical, biophysical, and physiological consequences. Annu Rev Biophys Biomol Struct 22:27–65.
26. Cantor CR, Schimmel PR. 1980. Biophysical chemistry part II: Techniques for the study of biological structure and function, 1st edn., San Francisco: W.H. Freeman & Company. p 503.
27. Arakawa T, Timasheff SN. 1982. Stabilization of protein structure by sugars. Biochemistry 21: 6536–6544.
28. Arakawa T, Kita Y, Carpenter JF. 1991. Protein–solvent interactions in pharmaceutical formulations. Pharm Res 8(3):285–291.
29. Pikal MJ. 1994. Freeze–drying of proteins: Process, formulation and stability. In: Cleland JL, Langer R, editors. Formulation and delivery of proteins and peptides, 1st edn., Washington D.C.: American Chemical Society. p 120–133.
30. Andya JD, Hsu CC, Shire SJ. 2003. Mechanisms of aggregate formation and carbohydrate excipient stabilization of lyophilized humanized monoclonal antibody formulations. Aaps Pharmsci 5(2):E10.
31. Carpenter JF, Pikal MJ, Chang BS, Randolph TW. 1997. Rational design of stable lyophilized protein formulations: Some practical advice. Pharm Res 14(8):969–975.
32. Carpenter JF, Chang BS, Garzon-Rodriguez W, Randolph TW. 2002. Rational design of stable lyophilized protein formulations: Theory and practice. Pharm Biotechnol 13:109–133.
33. Cleland JL, Lam X, Kendrick B, Yang J, Yang TH, Overcashier D, Brooks D, Hsu C, Carpenter JF. 2001. A specific molar ratio of stabilizer to protein is required for storage stability of a lyophilized monoclonal antibody. J Pharm Sci 90(3):310–321.
34. Genovesi CS. 1983. Several uses for tangential-flow filtration in the pharmaceutical industry. J Parenter Sci Technol 37(3):81–86.

35. Shiloach J, Martin N, Moes H. 1988. Tangential flow filtration. Adv Biotechnol Processes 8:97–125.
36. van Reis R, Zydney A. 2001. Membrane separations in biotechnology. Curr Opin Biotechnol 12(2):208–211.
37. Thomas CR, Nienow AW, Dunnill P. 1979. Action of shear on enzymes: Studies with alcohol dehydrogenase. Biotechnol Bioeng 21(12):2263–2278.
38. Watterson JG, Schaub MC, Waser PG. 1974. Shear-induced protein–protein interaction at the air–water interface. Biochimica et Biophysica Acta 356(2):133–143.
39. Gehlert G, Luque S, Belfort G. 1998. Comparison of ultra- and microfiltration in the presence and absence of secondary flow with polysaccharides, proteins, and yeast suspensions. Biotechnol Progress 14(6):931–942.
40. Masters K. 1985. Spray drying handbook, 4th edn., New York: John Wiley & Sons.
41. Rouan SKE. 1996. Biotechnology-based pharmaceuticals. In: Banker GS, Rhodes CT, editors. Modern pharmaceutics, 3rd edn., New York: Marcel Dekker. p 843–873.
42. Poupitch G. 1994. Fluid bed drying in the laboratory. Am Biotechnol Lab 12(4):30–34.
43. Wildfong PL, Samy AS, Corfa J, Peck GE, Morris KR. 2002. Accelerated fluid bed drying using NIR monitoring and phenomenological modeling: Method assessment and formulation suitability. J Pharm Sci 91(3):631–639.
44. Mattern M, Winter G, Kohnert U, Lee G. 1999. Formulation of proteins in vacuum-dried glasses. II. Process and storage stability in sugar-free amino acid systems. Pharm Dev Technol 4(2):199–208.
45. Miller DP, Anderson RE, de Pablo JJ. 1998. Stabilization of lactate dehydrogenase following freeze thawing and vacuum-drying in the presence of trehalose and borate. Pharm Res 15(8):1215–1221.
46. Avis KE. 1990. Parenteral preparations. In: Gennaro AR, editor Remington's pharmaceutical sciences, 18th edn., Easton, PA: Mack Publishing Company. p 1545–1569.
47. Harris LJ, Skaletsky E, McPherson A. 1995. Crystallization of intact monoclonal antibodies. Proteins 23(2):285–289.
48. Shenoy B, Wang Y, Shan W, Margolin AL. 2001. Stability of crystalline proteins. Biotechnol Bioeng 73(5):358–369.
49. Gatlin LA, Gatlin CAB. 1999. Formulation and administration techniques to minimize injection pain and tissue dammage associated with parenteral products. In: Gapta PK, Brazeau GA, editors. Injectable drug development: Techniques to reduce pain and irritation, 1st edn., Denver: Interpharm Press. p 401–421.
50. Jones AJS. 1993. Analytical methods for the assessment of protein formulations and delivery systems. In: Cleland JL, Langer R, editors. Formulation and delivery of peptides and proteins, 1st edn., Washington D.C.: American Chemical Society. p 22–45.
51. Pearlman R, Nguyen TH. 1990. Analysis of protein drugs. In: Lee VH, editor. Peptide and protein drug delivery, 1st edn., New York: Marcel Dekker, Inc. p 247–301.
52. Hatley RHM. 1990. International symposium on biological product freeze-drying and formulation, Bethesda MD. p 105–122.
53. Breen ED, Curley JG, Overcashier DE, Hsu CC, Shire SJ. 2001. Effect of moisture on the stability of a lyophilized humanized monoclonal antibody formulation. Pharm Res 18(9):1345–1353.
54. McPhillips H, Craig DQ, Royall PG, Hill VL. 1999. Characterization of the glass transition of HPMC using modulated differential scanning calorimetry. Int J Pharm 180:83–90.
55. Costantino HR, Chen B, Griebenow K, Hsu CC, Shire SJ. 1998. Fourier-transform infrared spectroscopic investigation of the secondary structure of aqueous and dried recombinant human deoxyribonuclease I. Pharm Pharmacol Commun 4:391–395.
56. Prestrelski SJ, Tedeschi N, Arakawa T, Carpenter JF. 1993. Dehydration-induced conformational transitions in proteins and their inhibition by stabilizers. Biophysical J 65(2):661–671.
57. Sharma VK, Kalonia DS. 2003. Steady-state trytophan fluorescence spectrscopy study to probe tertiary structure of proteins in solid powders. J Pharm Sci 92(4):890–899.
58. Shire SJ. 1992. Analytical ultracentrifugation and its use in biotechnology. In: Schuster TM, Laue TM, editors. Modern analytical ultracentrifugation, 1st edn., Boston: Birkhauser. p 261–297.
59. Lebowitz J, Lewis MS, Schuck P. 2002. Modern analytical ultracentrifugation in protein science: A tutorial review. Protein Sci 11(9):2067–2079.
60. Wen J, Arakawa T, Philo JS. 1996. Size-exclusion chromatography with on-line light-scattering, absorbance, and refractive index detectors for studying proteins and their interactions. Anal Biochem 240(2):155–166.
61. Moore JM, Patapoff TW, Cromwell ME. 1999. Kinetics and thermodynamics of dimer formation and dissociation for a recombinant humanized monoclonal antibody to vascular endothelial growth factor. Biochemistry 38(42):13960–13967.
62. Bloomfield VA. 1981. Quasi-elastic light scattering applications in biochemistry and biology. Annu Rev Biophys Bioeng 10:421–450.
63. Georgalis Y, Saenger W. 1999. Light scattering studies on supersaturated protein solutions. Sci Progress 82(Pt. 4):271–294.
64. Pecora R. 1972. Quasi-elastic light scattering from macromolecules. Annu Rev Biophys Bioeng 1:257–276.

65. Schurr JM. 1977. Dynamic light scattering of biopolymers and biocolloids. CRC Crit Rev Biochem 4(4):371–431.
66. Minton AP. 1989. Analytical centrifugation with preparative ultracentrifuges. Anal Biochem 176: 209–216.
67. Johnson ML, Straume M. 1994. Comments on the analysis of sedimentation equilibrium experiments. In: Schuster TM, Laue TM, editors. Modern analytical ultracentrifugation, 1st edn., Boston: Birkhauser. p 37–63.
68. Schuck P. 1998. Sedimentation analysis of noninteracting and self-associating solutes using numerical solutions to the Lamm equation. Biophys J 75(3):1503–1512.
69. Schuck P, Perugini MA, Gonzales NR, Howlett GJ, Schubert D. 2002. Size-distribution analysis of proteins by analytical ultracentrifugation: Strategies and application to model systems. Biophys J 82(2):1096–1111.
70. de la Torre JG. 1992. Sedimentation coefficients of complex biological particles. In: Harding SE, Rowe AJ, Horton JC, editors. Analytical ultracentrifugation in biochemistry and polymer science, 1st edn., Cambridge, England: The Royal Society of Chemistry. p 333–345.
71. Liu J, Lester P, Builder S, Shire SJ. 1995. Characterization of complex formation by humanized anti-IgE monoclonal antibody and monoclonal human IgE. Biochemistry 34(33):10474–10482.

Crystalline monoclonal antibodies for subcutaneous delivery

Mark X. Yang, Bhami Shenoy, Matthew Disttler, Reena Patel, Margaret McGrath, Sergey Pechenov, and Alexey L. Margolin*

Altus Biologics, Inc., 625 Putnam Avenue, Cambridge, MA 02139

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Therapeutic applications for mAbs have increased dramatically in recent years, but the large quantities required for clinical efficacy have limited the options that might be used for administration and thus have placed certain limitations on the use of these agents. We present an approach that allows for s.c. delivery of a small volume of a highly concentrated form of mAbs. Batch crystallization of three Ab-based therapeutics, rituximab, trastuzumab, and infliximab, provided products in high yield, with no detectable alteration to these proteins and with full retention of their biological activity *in vitro*. Administration s.c. of a crystalline preparation resulted in a remarkably long pharmacokinetic serum profile and a dose-dependent inhibition of tumor growth in nude mice bearing BT-474 xenografts (human breast cancer cells) *in vivo*. Overall, this approach of generating high-concentration, low-viscosity crystalline preparations of therapeutic Abs should lead to improved ease of administration and patient compliance, thus providing new opportunities for the biotechnology industry.

Because of their successful use in targeting distinct antigens, mAbs have become powerful therapeutic agents in the treatment of cancer, inflammatory, cardiovascular, respiratory, and infectious diseases. It has been estimated that >20% of all biopharmaceuticals currently being evaluated in clinical trials are mAbs. In general, mAb therapies require the delivery of between 100 mg and 1 g of protein per dose. Because the high end of formulation concentrations for mAbs is typically in the range of 50 mg/ml, such treatments commonly require the administration of 2–20 ml. Typically, such volumes can be given only through i.v. infusion performed in a clinical or hospital setting. A broadly applicable method of achieving the high-concentration mAb preparations required to deliver these large protein doses in a small volume appropriate for s.c. injection will likely lead to an expansion of therapeutic opportunities and an increase in patient compliance. One approach would be merely to prepare extremely high-concentration preparations of soluble mAbs, on the order of 200–250 mg/ml. However, such highly concentrated solutions often result in very high viscosity, protein aggregation, and poor overall stability. We have addressed this problem by identifying methods to prepare pharmaceutically acceptable crystalline formulations of mAbs, using several of those currently in clinical use. The therapeutic mAbs used in our studies target the cell-surface antigen CD20 (1), tumor necrosis factor α (TNF-α) (2), and human epidermal growth factor receptor 2 (HER2) (3).

To the best of our knowledge, crystalline full-length mAbs have not been explored for therapeutic use. Among ≈400 biopharmaceuticals that are either approved or in advanced clinical trials, there is only one product, insulin, that is produced and administered in crystalline form (4). Yet crystallization of macromolecule pharmaceuticals, particularly proteins, can offer significant advantages, such as high stability, streamlining of manufacturing, controlled release of activity, and high doses at the delivery site, which is especially attractive for mAb therapies (5–7). Although crystallization of full-length Abs has been a subject of significant interest for the last three decades, very few intact mAbs have ever been crystallized (8–10). The difficulty in mAb crystallization largely has been attributed to their large size, the presence of surface oligosaccharides, and a high degree of segmental flexibility. All of the previously reported methods of crystallizing mAbs have used the vapor diffusion technique. This approach yields only minute quantities of crystals, is designed to produce large crystals required for x-ray studies, and commonly uses agents unacceptable for use in humans.

We have devised batch-process methods to produce crystalline suspensions of three approved therapeutic Abs: rituximab, trastuzumab, and infliximab. Rituximab is a chimeric IgG1 κ Ig that binds the CD20 antigen on the surface of normal and malignant B lymphocytes and is used in the treatment of non-Hodgkin's lymphoma (11). Trastuzumab is a humanized IgG1 κ Ig used to treat breast cancer through a mechanism that presumably involves binding to the human epidermal growth factor receptor 2 (HER2) overexpressed on these tumor cells (12). Infliximab is a chimeric IgG1 κ Ig that binds to the soluble and transmembrane forms of TNF-α for the treatment of rheumatoid arthritis and Crohn's disease (13). The methods we have developed for crystallization provide crystals in high yield that show excellent physical and chemical stability as well as retention of biological activity *in vitro*. Furthermore, we have examined the *in vivo* s.c. injection of crystalline trastuzumab and infliximab suspensions. The low-viscosity, highly concentrated formulations of crystalline mAbs demonstrated an extended serum pharmacokinetic (PK) profile and high bioavailability compared with the soluble mAbs delivered i.v. Finally, we have demonstrated that the crystalline formulation of trastuzumab was effective in a preclinical model of human breast cancer.

Materials and Methods

Materials. Rituximab, commercially available as Rituxan, and trastuzumab, commercially available as Herceptin, were from Genentech (South San Francisco, CA). Infliximab, commercially available as Remicade, was from Centocor. A Biosep-SEC-S-3000 HPLC gel filtration column was from Phenomenex (Torrance, CA). The Bradford protein assay reagent was from Bio-Rad. All other chemicals were reagent-grade.

General Methods. The protein content of samples was determined by using the Bio-Rad protein assay reagent. The crystal integrity of the proteins in the formulations was measured by comparing the size and shape of the crystals with those in mother liquor by qualitative microscopic observations. To demonstrate purity and integrity of mAbs before and after crystallization, the following techniques were used: SDS/PAGE, capillary isoelectrofocusing, size exclusion column (SEC)-HPLC, dynamic light scattering, MS, peptide mapping, and N-terminal sequencing. In addition, the total carbohydrate and monosaccharide composition and N-linked oligosaccharide profiling were determined by using Bio-Rad kits (see *Methods* and *Results* in *Supporting Text*, which

Abbreviations: TNF-α, tumor necrosis factor α; PK, pharmacokinetic; PEG, polyethylene glycol.

*To whom correspondence should be addressed. E-mail: margolin@altus.com.

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Structure of Carbohydrate on mAb. The glycan structure of rituximab was determined by using capillary electrophoresis (CE) with laser-induced fluorescence detection after digestion with various glycosidases (14). The released glycans were analyzed after derivatization with 8-aminopyrene-1,3,6-trisulfonic acid, trisodium salt (APTS) on a Beckman Coulter P/ACE MDQ CE system equipped with an argon-ion laser with an excitation wavelength of 488 nm and an emission band-pass filter of 520 ± 10 nm. Sequential enzymatic digestion, electrophoretic mobility, and monosaccharide composition, as well as the molecular weight of the glycans released by liquid chromatography (LC)/MS, were used to get the complete structure.

Rituximab Direct Cytotoxicity Assay. Direct cytotoxicity measures the intrinsic toxic effect of an Ab on the target cell by coincubating the target cells with different concentrations of the Ab. Cell viability is counted after coincubation with the Ab. RAJI lymphoma cells (ATCC no. CCL-86) were cultured in RPMI medium 1640 supplemented with 10% FBS and diluted to 0.5 ×

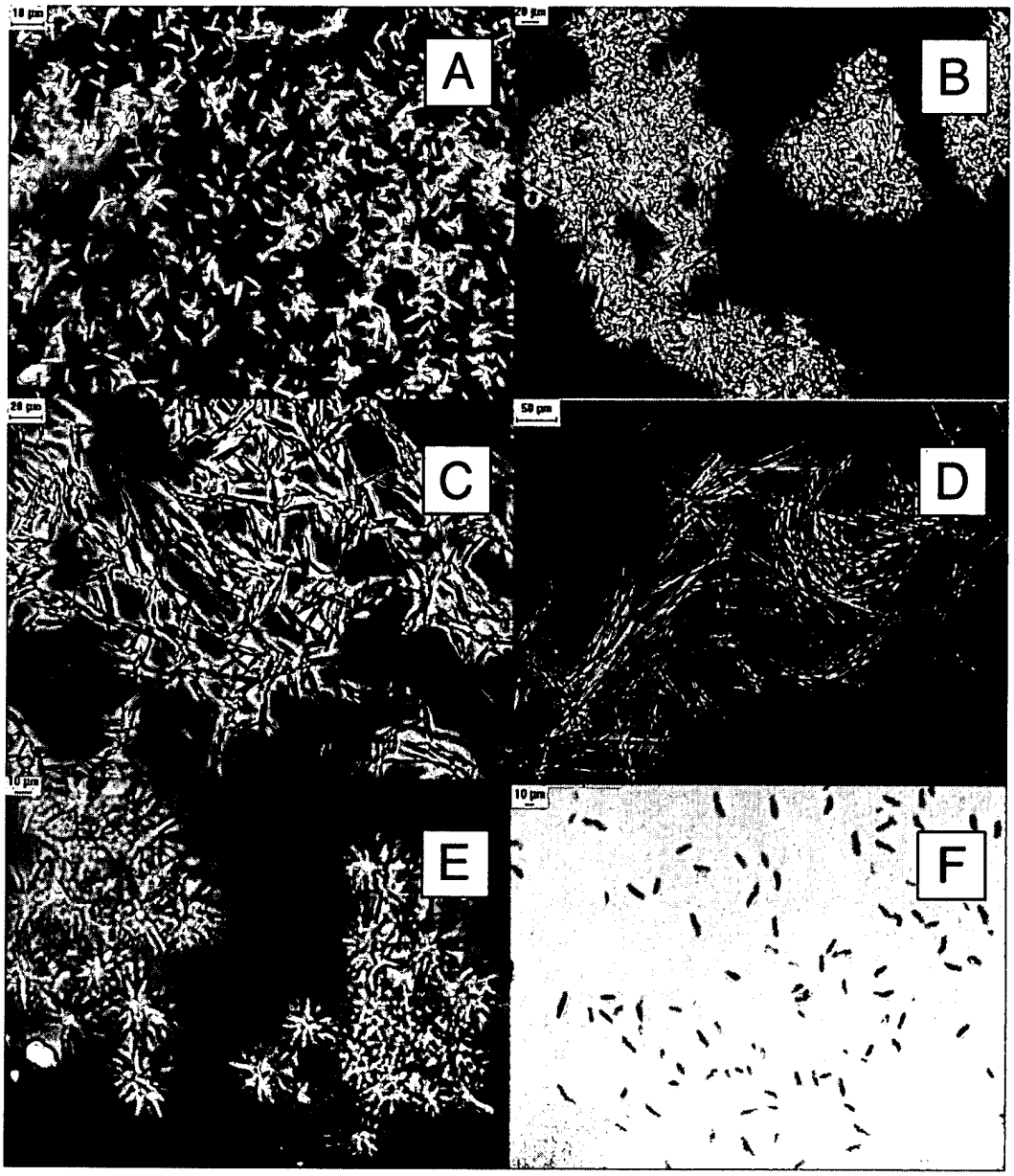

Fig. 1. Crystals of rituximab (*A* and *B*), trastuzumab (*C* and *D*), and infliximab (*E* and *F*). Crystallization protocols are described elsewhere (15). Crystals were produced in 400-μl batches with the following precipitants: 12% polyethylene glycol (PEG) 400, 1.17 M Na_2SO_4 in 100 mM Hepes buffer, pH 7.7 (*A*); 35% PEG 400, 0.2 M $CaCl_2$ in 0.1 M Hepes buffer, pH 7.5 (*B*); 25% PEG 400, 5% PEG 8000, 10% propylene glycol, 0.1% Tween 80 in Tris buffer, pH 8.5 (*C*); 20% PEG 400, 10% PEG 8000, 10% glycerol in 0.1 M Tris buffer, pH 7.0 (*D*); 20% PEG 300, 5% PEG 8000, 10% glycerol in 0.1 M Tris buffer, pH 7.0 (*E*); and 0.2 M NaCl, 10% isopropanol in 0.1 M Hepes buffer, pH 7.6 (*F*). Crystals were examined under an Olympus BX60 microscope equipped with a DXC-970MD 3CCD color video camera with camera adapter (CMA D2) and analyzed with IMAGE-PRO PLUS software (Media Cybernetics, Silver Spring, MD). Samples of protein crystals were covered with a glass coverslip, mounted, and examined under ×10 magnification by using an Olympus microscope with an Olympus UPLAN F1 objective lens ×10/0.30 PH1 (phase contrast).

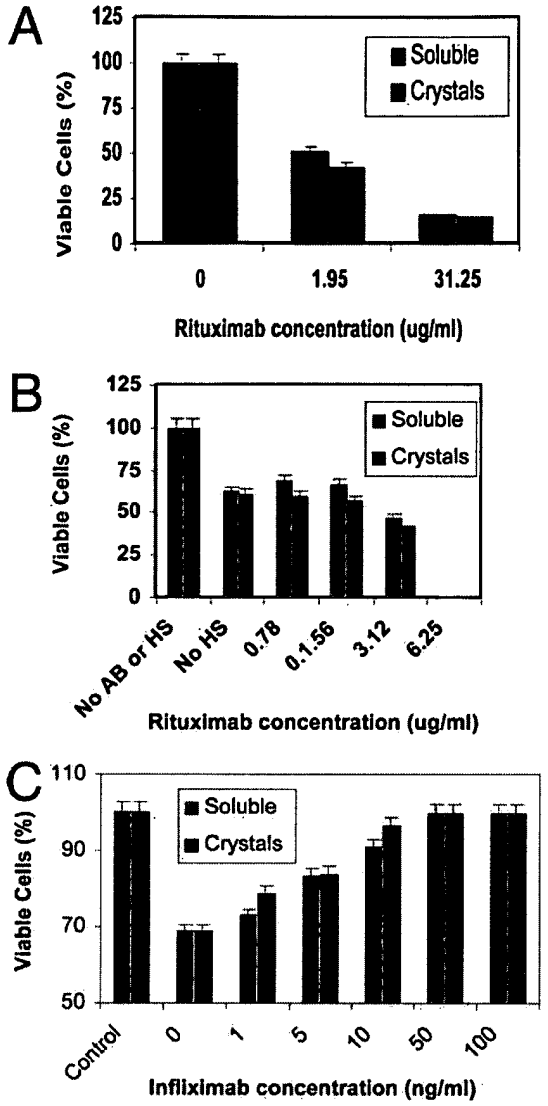

Fig. 2. *In vitro* bioactivity of rituximab. Cultured RAJI lymphoma cells were detached, diluted to 0.5 × 10^5 cells per ml, and added to 96-well plates (100 μl per well). (*A*) Direct cytotoxicity studies were performed by incubating RAJI lymphoma cells for 3 days in the presence of various concentrations of rituximab or dissolved crystals of rituximab (added in 25-μl volumes). (*B*) Complement-dependent cytotoxicity was determined by exposing a mixture of RAJI lymphoma cells and rituximab preparations (as in *A*), followed by the addition of various concentrations of human serum. (*C*) *In vitro* bioactivity of infliximab. Cultured L-929 mouse fibroblast cells were detached, diluted to 2 × 10^5 cells per ml, and added to 96-well plates (100 μl per well). TNF-α neutralization assays were performed by incubating mouse fibroblast cells overnight in the presence of various concentrations of infliximab or dissolved crystals of infliximab. The number of viable cells was determined by using a CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega).

10^5 cells per ml. A 100-μl aliquot of that culture was transferred to 1 well of a 96-well plate and cultured in the presence of various concentrations of native (soluble) and dissolved crystals of rituximab for 3 days. The number of viable cells remaining after the 3-day incubation was determined by using CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega).

Rituximab Complement-Dependent Cytotoxicity Assay. RAJI lymphoma cells were cultured in growth media and diluted to 0.5 × 10^5 cells per ml. A 100-μl aliquot of this culture was transferred

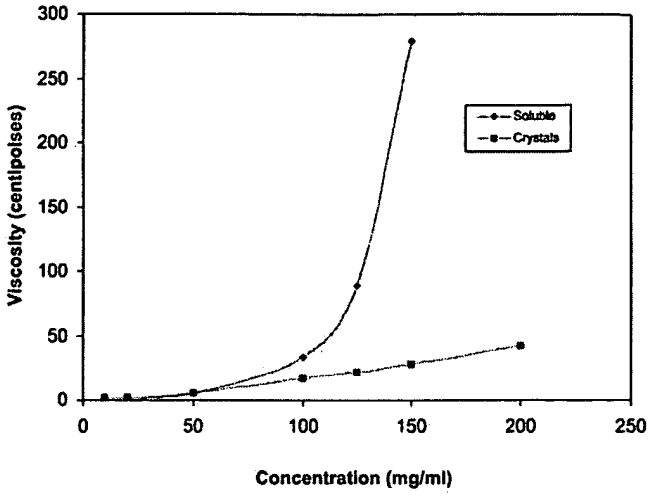

Fig. 3. Viscosity measurements of infliximab. The viscosity of soluble and crystalline suspensions of infliximab was measured by using the Cannon-Fenske (State College, PA) viscometer according to the manufacturer's instructions. Commercially available infliximab [100 mg per vial containing 500 mg of sucrose, 0.5 mg of polysorbate 80, 2.2 mg of monobasic sodium phosphate (monohydrate), and 6.1 mg of dibasic sodium phosphate (dihydrate)] was reconstituted in water to concentrations of 10, 25, 50, 100, 125, and 150 mg/ml and compared with various concentrations of crystalline suspensions. The excipients in the commercial lyophilized formulation did not contribute significantly to the viscosity of the reconstituted infliximab. Even at 150 mg/ml, the viscosity of the vehicle was only 26 centipoise (cps; 1 cps = 10^{-3} Pa·sec), compared with 275 cps for soluble infliximab. For crystalline suspensions of infliximab, a 200 mg/ml solution (which was in formulation buffer containing 10% ethanol, 10% PEG 3350, 0.1% Tween 80, and 50 mM trehalose in 50 mM sodium phosphate buffer, pH 7.0) was tested for viscosity in addition to the concentrations mentioned above for soluble infliximab.

to 1 well of a 96-well plate and cultured in the presence of 25 μg/ml native or dissolved crystals of rituximab and various concentrations of human serum (as a source of complement system) for 3 days. The number of viable cells remaining after the 3-day incubation was determined with the CellTiter assay as before.

Infliximab TNF-α Neutralization Assay. TNF-sensitive L-929 mouse fibroblast cells (ATCC no. CCL-1), which were cultured in Eagle's minimal essential medium (EMEM) supplemented with 10% horse serum, were detached from the culture flask by trypsin digestion and diluted to 2 × 10^5 cells per ml. A 100-μl aliquot of the culture was transferred to 1 well of a 96-well plate and incubated at 37°C with a 5% carbon dioxide-in-air atmosphere overnight. After aspiration off the medium, 100 μl of fresh medium, supplemented with 1 μg/ml actinomycin D, 100 pg/ml TNF-α, and various concentrations of native (soluble) and dissolved infliximab crystals, was added to each well and incubated overnight before the number of viable cells was determined.

Infliximab PK Studies in Rats. Forty to 45 BBDR/Wor male rats, weighing between 200 and 250 g, were used for the study, and infliximab was given through either s.c. or i.v. routes. On day 1, rats were weighed and then given a single i.v. or s.c. injection of infliximab (8 or 80 mg/kg, at concentrations of 5 ml/kg of body weight). Blood samples were taken at 0.5, 2, 4, 6, 8, 24, 48, 72, 96, 120, 168, 336, 504, and 672 h after dosing. Blood samples were taken at the additional time points of 2 and 10 min after dosing for the i.v. group. At each time point, six rats were bled from the tail, and ≈100 μl of whole blood was collected. The collected

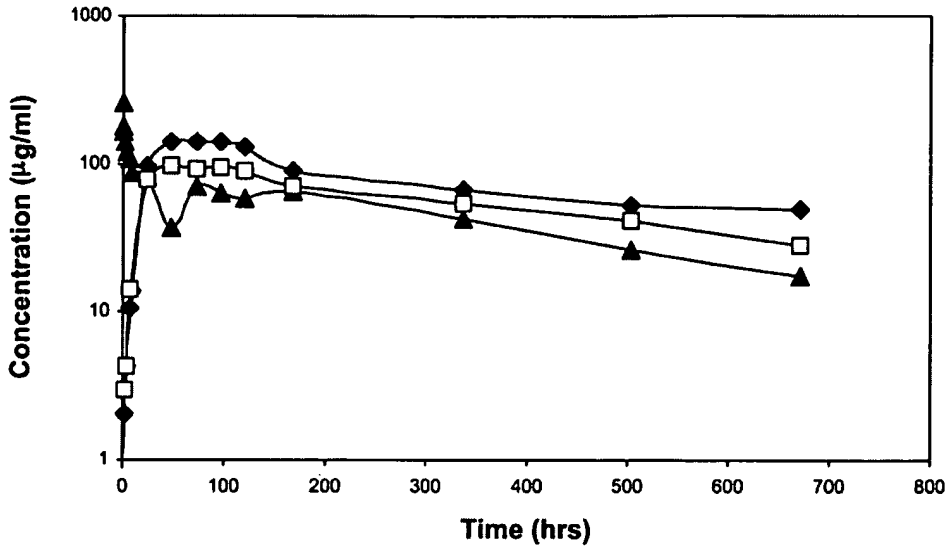

Fig. 4. PK studies of infliximab. Approximately 100 μl of infliximab (20 mg/ml) was administered to BBDR/Wor rats having a body weight of ≈250 g to provide a dose of 8 mg/kg. The mAb was administered in a soluble form either i.v. (▲) or s.c. (□) or as a crystalline suspension s.c. (◆). The maximum concentration (C_{max}) for soluble infliximab was 257 μg/ml for the i.v. sample and 95 and 112 μg/ml for the s.c. soluble and crystalline samples, respectively. The half-life of soluble infliximab was 270 h for the i.v. sample; the half-life of the s.c. soluble and crystalline infliximab was 390 and 779 h, respectively. The total area under the curve (AUC_{0-t}) for soluble infliximab was 29.3 mg·h·ml^{-1} for the i.v. sample and 37.5 and 42.8 mg·h·ml^{-1} for the soluble and crystalline s.c. samples, respectively.

blood was processed as described above, and the levels of infliximab were determined by ELISA.

Infliximab ELISA Protocol. The wells of 96-well high-binding polystyrene plates were coated with 50 μl per well of 10 μg/ml anti-human Ab (Pierce) at 4°C overnight. The anti-human Ab was removed, and the plates were washed three times with a buffer containing 50 mM Tris (pH 8.0), 0.138 M sodium chloride, 0.0027 M potassium chloride, and 0.05% Tween 20 (TBST). After blocking with 3% nonfat dry milk in TBST for 2 h at room temperature, a 100-μl aliquot of control (either saline or nonspecific IgG) or a diluted serum sample containing infliximab was added to each well and incubated for 2 h at room temperature. After washing with TBST three times, a 100-μl aliquot of horseradish peroxidase-conjugated anti-human Ab (1:25,000 dilution with nonfat dry milk in TBST) then was added to each well, and the plates were incubated for 1 h. The color reaction was initiated by adding 100 μl of 3,3′,5,5′-tetramethylbenzidine substrate to each well and was allowed to proceed for 30 min at room temperature in the dark before it was stopped with 100 μl of 0.5 M sulfuric acid. The optical density was read at a wavelength of 450 nm (OD_{450}) on an automatic microplate reader. The OD_{450} values, which corresponded to the amount of infliximab in the blood sample tested, then were plotted.

Histochemistry of Crystalline Trastuzumab in BALB/c Mice. After 12, 24, 48, and 504 h after injection of 0.1 ml of 200 mg/ml trastuzumab crystals or carrier buffer (negative control) or lipopolysaccharide (LPS purified from *Escherichia coli*; 50 μg in 0.1 ml of saline; positive control), the injection sites were isolated for necropsy. Any gross pathology or evidence of formulation at the injection site was noted for each of the three mice per group. A portion of the skin was fixed in neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin/eosin for histology examination for inflammatory responses. The skin of the injection site was subdivided into epidermis, dermis, and s.c. panniculus to make evaluation more concise. There were no treatment-related microscopic effects. All tissues from treated animals [skin (at injection site), brain, bladder, eyes, brown fat, white fat, testes, epididymis, heart, stomach, small

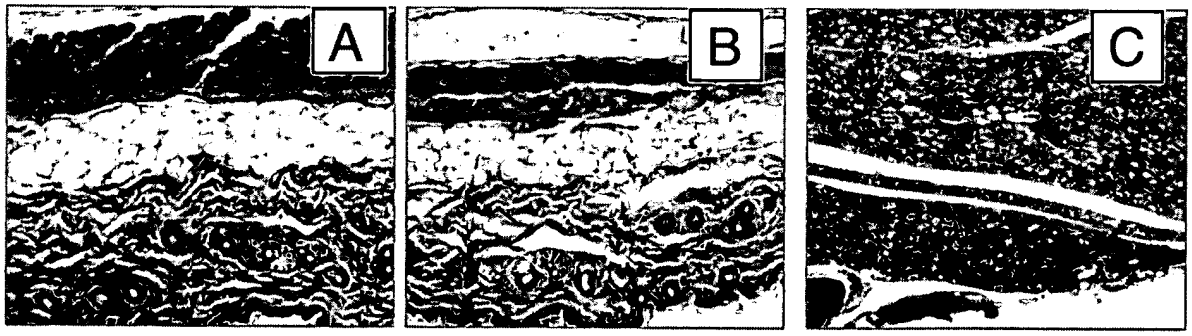

Fig. 5. s.c. injection site analysis for trastuzumab. Injection sites were isolated at necropsy 12 h after the injection of 0.1 ml of 200 mg/ml trastuzumab crystals in a formulation buffer, which is different from the mother liquor, containing 10% ethanol and 10% PEG 3350 (800 mg/kg of body weight; *A*), 0.1 ml of a 7.5 mg/ml soluble commercial formulation of trastuzumab (30 mg/kg; *B*), or lipopolysaccharides (LPS; positive control; *C*). A portion of the skin at the site of injection was fixed in neutral buffered formalin oriented with the internal surface on a cassette-size square of paper, embedded in paraffin, and sectioned for histology. Paraffin sections were processed through hematoxylin/eosin stain. (All micrographs shown are ×40.)

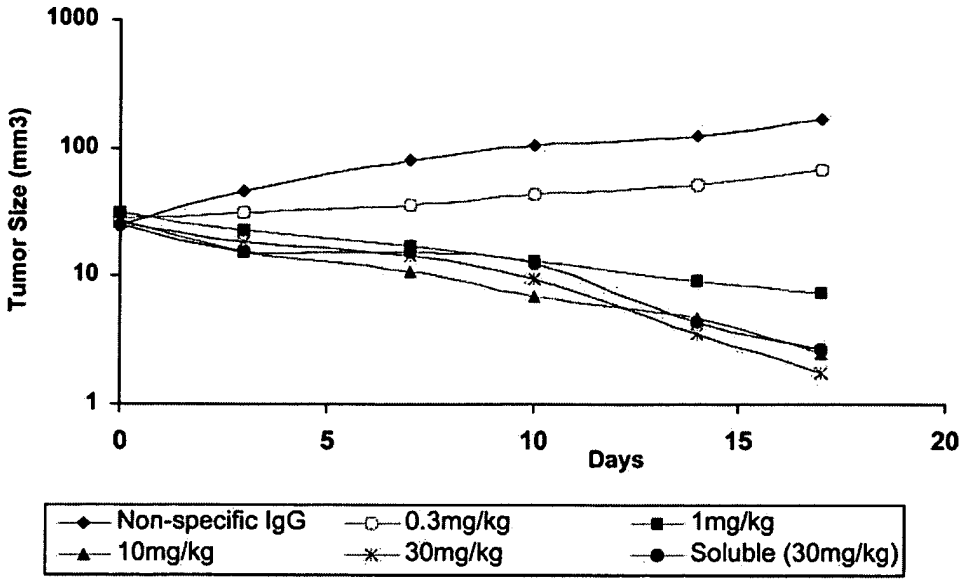

Fig. 6. *In vivo* bioactivity/efficacy of trastuzumab in nude mice. Human breast cancer BT-474 cells were used to establish tumor nodules that could be monitored by measuring their dimensions every 3–4 days with vernier calipers. Animals were placed randomly into treatment groups ($n = 6$). Tumor volume was calculated by the formula $\pi/6 \times$ [larger diameter $\times$ (smaller diameter)^2]. Ab treatments were initiated when tumors became >20–30 mm^3 in size in one set of animals. Crystalline trastuzumab suspensions and control solutions (containing a nonspecific IgG also at 30 mg/kg) were administered s.c. in 10 doses over 5 weeks (2 doses per week). Mice then were killed for pathological and histopathological examination and compared with the untreated animals (not injected with BT-474 cells). The mean of each experimental group is shown. Statistical significance of data comparing vehicle to anti- human epidermal growth factor receptor 2 (HER2) was determined by using one-way ANOVA software (Microsoft EXCEL).

intestine, large intestine, kidneys, liver, lungs, pancreas, thyroid, spinal cord, spleen, lymph nodes (cervical), thymus, esophagus, bone (femur), skeletal muscles (including psoas)] were found to be within normal limits and/or had incidental findings noted equally in controls and known to be common incidental findings for this age, sex, and strain of mouse.

Trastuzumab Efficacy Study in Nude Mice. Human breast cancer BT-474 cells, which overexpress human epidermal growth factor receptor 2 (HER2) on their surface and are inhibited by anti-HER2 Ab, from ATCC were cultured in DMEM supplemented with 10% FBS, 10% NCTC 109 medium, and 1.2 mM oxaloacetic acid. After a few cell passages, the human breast cancer cells were inoculated s.c. (5×10^7 cells per animal) in the hind thighs of 3-month-old female athymic mice. Before inoculation, mice were primed for 10–14 days with 17β-estradiol applied s.c. in a biodegradable carrier-binder (1.7 mg of estradiol per pellet) to promote growth of the estrogen-dependent breast cancer cells. Crystalline trastuzumab suspensions and control solutions (nonspecific IgG) were administered s.c. in 10 doses over 5 weeks. The tumor nodules were measured every 3–4 days with vernier calipers. Mice were killed and processed for pathological and histopathological examination at the end of the treatment.

Results and Discussion

Three commercially available therapeutic mAbs, rituximab, trastuzumab, and infliximab, were successfully crystallized in large batches. Our batch crystallization protocols for these three mAbs resulted in yields of >90% for rituximab and >85% for trastuzumab and infliximab (Fig. 1). Manipulation of these protocols resulted in a variety of crystal morphologies that included needles and rice-shaped and star clusters. Analytical characterization of crystalline suspensions upon dissolution at 25°C was performed by using SDS/PAGE, isoelectrofocusing, dynamic light scattering, matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) MS, peptide mapping, N-terminal sequencing, and carbohydrate analysis (see Figs. 7–16, which are published as supporting information on the PNAS web site). These studies showed no differences in purity and integrity between the soluble protein currently administered clinically (used in the preparation of our crystalline suspensions) and protein obtained after dissolution of these crystalline suspensions. The crystalline mAbs were dissolved easily in either water or saline in less than a minute. Moreover, crystalline samples of rituximab (10 mg/ml in mother liquor at room temperature) and trastuzumab (22 mg/ml in mother liquor at 4°C) exhibited complete retention of native state as determined by size exclusion column HPLC (no aggregation or fragments) after 12 months of storage at room temperature and 20 weeks at 4°C, respectively. The bioactivity of crystalline mAb preparations was evaluated in both direct and complement-dependent cytotoxicity tests (1) by using RAJI lymphoma cells *in vitro* for rituximab (Fig. 2 *A* and *B*) and in a TNF-α neutralization assay in the cultured fibroblast cells (Fig. 2*C*) for infliximab. Both tests demonstrated a similar bioactivity of mAbs before and after crystallization.

To determine whether mother liquor could be replaced with another pharmaceutically acceptable vehicle, a number of injectable vehicles were tested. We found that the PEG/ethanol mixtures are useful in providing low-viscosity formulations that maintain both crystallinity and integrity of mAbs for a long time. For example, a 200 mg/ml suspension of crystalline trastuzumab in PEG/ethanol did not show any aggregation, as assessed by size exclusion column HPLC over a period of 20 weeks at 4°C. Normally, protein solutions become quite viscous at concentrations of >100 mg/ml (S. J. Shire, unpublished communication). Yet, even at 200 mg/ml, the viscosity of the crystalline suspensions of infliximab (Fig. 3) and trastuzumab remained low and allowed for easy injection with a 26-gauge needle. (The time required to pass a 1-ml suspension of infliximab was <20 sec; for trastuzumab it was only 5 sec.) To provide a uniform suspension

for accurate dosing, the crystalline formulations were mixed by inverting the vials before injection.

Crystalline infliximab injected s.c. into BBDR/Wor rats at a dose of 8 mg/kg was found to produce an extended serum PK profile compared with similar s.c. or i.v. injections with the commercially prepared soluble form of this mAb (Fig. 4). The crystalline mAb appeared to take longer to enter the bloodstream, achieving a lower calculated maximum concentration (C_{max}) value (112 μg/ml, compared with 280 μg/ml injected by i.v. route) and significantly longer (2.9×) half-life. In comparison with i.v. injections, s.c. injections of crystalline preparations of mAbs resulted in an apparent increase in total area under the curve (AUC_{0-t}; 42.8 mg·h·ml^{-1} s.c. vs. 29.6 mg·h·ml^{-1} i.v.), thus indicating high bioavailability. More extensive PK studies will be necessary to determine whether the apparent increase in the AUC is due to the properties of crystalline suspensions or s.c. route of administration (16), or whether it simply reflects variance in sample analysis. At the same time, we have demonstrated the integrity of the native Abs in blood samples by Western blotting as well as by matrix-assisted laser desorption ionization/time-of-flight analysis (data not shown). The results indicated that there were no Ab degradation products that would have contributed to higher ELISA values and thereby artificially increased the AUC.

Injection site assessment was performed 12, 24, 48, and 504 h after s.c. injection of 0.1 ml of a 200 mg/ml crystalline trastuzumab suspension into BALB/c mice. The results for a 12-h time period for crystalline trastuzumab (800 mg/kg), soluble trastuzumab (30 mg/kg) and lipopolysaccharides (LPS) are shown in Fig. 5 *A*, *B*, and *C*, respectively. Crystalline and soluble protein had minimal effects over the same time period, whereas LPS, used as a positive control, had the highest incidence and the greatest severity grades for mononuclear infiltration at the injection site. Histological analysis of injection sites of animals dosed with crystalline suspensions of mAbs did not reveal residual crystals after 12 h, suggesting a rapid dissolution of crystals within the s.c. space. In addition, all tissues from treated animals were found to be within normal limits and/or had incidental findings noted equally in controls and known to be common incidental findings for this age, sex, and strain of mouse.

The efficacy of crystalline trastuzumab was examined in a series of experiments conducted in nude mice bearing BT-474 xenografts (17). Crystalline suspensions of trastuzumab, given s.c. at doses from 1 to 30 mg/kg (0.1 ml) twice a week for 4 weeks, induced a dose-dependent inhibition of growth of the BT-474 xenografts (Fig. 6). More pronounced antitumor activity was seen at doses >1 mg/kg, with complete tumor eradication in two of six mice treated at 10 mg/kg and in three of six mice treated with 30 mg/kg. The magnitude of the effects was similar to those achieved by i.v. injection of the soluble trastuzumab (18). All tumors in the control group receiving a nonspecific IgG enlarged during this period (Fig. 6). Crystalline Ab suspensions were well tolerated and comparable with those of soluble Ab formulations.

In summary, our data support the potential application of crystalline suspensions of mAb therapeutics for the generation of high-concentration, low-viscosity formulations for s.c. administration that rapidly dissolve after injection. This approach is commercially feasible; proteins can be batch-crystallized with good yields into a range of small crystals that demonstrate excellent physical/chemical protein stability upon storage with full retention of biological activity. Once crystallized, mAbs can be concentrated into high-concentration suspensions that are biologically compatible when injected s.c. The s.c. administration of crystalline mAbs is efficacious and potentially can reduce the frequencies of dosing. Our data suggest that the application of crystalline suspensions for the administration of therapeutic mAbs can provide a great benefit to the biotechnology industry when a high concentration of delivered protein is desired. For example, mAb therapy often can be efficacious and safe for chronic therapies, but patient compliance for i.v. infusions at a clinic can limit these uses. Additionally, some mAbs are being examined in combination therapies, often with new, orally active drugs that would make frequent visits to a clinic for an i.v. infusion unacceptable. Overall, our studies show that crystalline suspensions provide an improved method of delivery for mAbs that would otherwise be difficult to administer via the s.c. route.

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1. Flieger, D., Renoth, S., Beier, I., Sauerbruch, T. & Schmidt-Wolf, I. (2000) *Cell. Immunol.* **204**, 55–63.
2. Siegel, S. A., Shealy, D. J., Nakada, M. T., Le, J., Woulfe, D. S., Probert, L., Kollias, G., Ghrayeb, J., Vilcek, J. & Daddona, P. E. (1995) *Cytokine* **7**, 15–25.
3. Pietras, R. J., Poen, J. C., Gallardo, D., Wongvipat, P. N., Lee, H. J. & Slamon, D. J. (1999) *Cancer Res.* **59**, 1347–1355.
4. Brange, J. & Volund, A. (1999) *Adv. Drug Delivery Rev.* **35**, 307–335.
5. Shenoy, B., Wang, Y., Shan, W. & Margolin, A. L. (2001) *Biotechnol. Bioeng.* **73**, 358–369.
6. Margolin, A. L. & Navia, M. A. (2001) *Angew. Chem. Int. Ed. Engl.* **40**, 2204–2222.
7. Jen, A. & Merkle, H. P. (2001) *Pharm. Res.* **18**, 1483–1488.
8. Harris, L. J., Larson, S. B. & McPherson, A. (1999) *Adv. Immunol.* **72**, 191–208.
9. Kuznetsov, Y. G., Day, J., Newman, R. & McPherson, A. (2000) *J. Struct. Biol.* **131**, 108–115.
10. Saphire, E. O., Parren, P. W., Barbas, C. F., III, Burton, D. R. & Wilson, I. A. (2001) *Acta Crystallogr. D* **57**, 168–171.
11. Hainsworth, J. D. (2002) *Semin. Oncol.* **29**, 1 Suppl. 2, 25–29.
12. Leyland-Jones, B. & Smith, I. (2001) *Oncology* **61**, Suppl. 2, 83–91.
13. Keating, G. M. & Perry, C. M. (2002) *BioDrugs* **16**, 111–148.
14. Ma, S. & Nashabeh, W. (1999) *Anal. Chem.* **71**, 5185–5192.
15. Shenoy, B., Govardhan, C. P., Yang, M. & Margolin, A. L. (2002) Patent Cooperation Treaty WO 02/072636 A2.
16. Ghetie, V. & Ward, K. S. (1997) *Immunol. Today* **18**, 592–598.
17. Colbern, G. T., Hiller, A. J., Musterer, R. S., Working, P. K. & Henderson, I. C. (1999) *J. Inorg. Biochem.* **77**, 117–120.
18. Baselga, J., Norton, L., Albanell, J., Kim, Y. M. & Mendelsohn, J. (1998) *Cancer Res.* **58**, 2825–2831.

Phase II Study of Weekly Intravenous Recombinant Humanized Anti-p185^{HER2} Monoclonal Antibody in Patients With HER2/*neu*-Overexpressing Metastatic Breast Cancer

By José Baselga, Debasish Tripathy, John Mendelsohn, Sharon Baughman, Christopher C. Benz, Lucy Dantis, Nancy T. Sklarin, Andrew D. Seidman, Clifford A. Hudis, Jackie Moore, Paul P. Rosen, Thomas Twaddell, I. Craig Henderson, and Larry Norton

Purpose: **Breast cancer frequently overexpresses the product of the HER2 proto-oncogene, a 185-kd growth factor receptor (p185^{HER2}). The recombinant humanized monoclonal antibody (rhuMAb) HER2 has high affinity for p185^{HER2} and inhibits the growth of breast cancer cells that overexpress HER2. We evaluated the efficacy and toxicity of weekly intravenous administration of rhuMAb HER2 in patients with HER2-overexpressing metastatic breast cancer.**

Patients and Methods: **We treated 46 patients with metastatic breast carcinomas that overexpressed HER2. Patients received a loading dose of 250 mg of intravenous rhuMAb HER2, then 10 weekly doses of 100 mg each. Patients with no disease progression at the completion of this treatment period were offered a maintenance phase of 100 mg/wk.**

Results: **Study patients had extensive metastatic disease, and most had received extensive prior anticancer therapy. Adequate pharmacokinetic levels of rhuMAb HER2 were obtained in 90% of the patients. Toxicity was minimal and no antibodies against rhuMAb HER2 were detected in any patients. Objective responses were seen in five of 43 assessable patients, and included one complete remission and four partial remissions (overall response rate, 11.6%; 95% confidence interval, 4.36 to 25.9). Responses were observed in liver, mediastinum, lymph nodes, and chest wall lesions. Minor responses, seen in two patients, and stable disease, which occurred in 14 patients, lasted for a median of 5.1 months.**

Conclusion: **rhuMAb HER2 is well tolerated and clinically active in patients with HER2-overexpressing metastatic breast cancers that had received extensive prior therapy. This is evidence that targeting growth factor receptors can cause regression of human cancer and justifies further evaluation of this agent.**

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DURING THE LAST DECADE, proto-oncogenes that encode growth factors and growth factor receptors have been found to play important roles in the pathogenesis of several human malignancies, including breast cancer.[1] The HER2 gene (also known as *neu* and as *c-erbB*-2) encodes a 185-kd transmembrane glycoprotein receptor (p185^{HER2}) that has partial homology with the epidermal growth factor receptor, and that shares with that receptor intrinsic tyrosine kinase activity.[2-4] HER2 is overexpressed in 25% to 30% of human breast cancers[5,6] and predicts for a worse prognosis in patients with primary disease that involves axillary lymph nodes.[5,7,8] Several lines of evidence support a direct role for HER2 in the pathogenesis and clinical aggressiveness of HER2-overexpressing tumors: The introduction of HER2 into non-neoplastic cells causes their malignant transformation.[9,10] Transgenic mice that express HER2 develop mammary tumors.[11] HER2 overexpression is common in ductal carcinomas in situ and in their associated invasive cancers.[12,13] Antibodies directed at p185^{HER2} can inhibit the growth of tumors and of transformed cells that express high levels of this receptor.[14-18]

The latter observation suggests that p185^{HER2} may be a potential target for the treatment of breast cancer or preinvasive breast lesions because these cells commonly overexpress HER2. The murine monoclonal antibody (MAb) 4D5, directed against the extracellular domain of p185^{HER2} (ECD^{HER2}), is a potent inhibitor of growth, in vitro and in xenograft models, of human breast cancer cells that overexpress HER2.[19-21] However, murine antibodies are limited clinically because they are immunogenic. To facilitate further clinical investigations, therefore, MAb 4D5 was humanized. The resulting recombinant humanized anti-p185^{HER2} monoclonal antibody (rhuMAb HER2) was found to be safe and to have dose-dependent pharmacokinetics in two prior phase I clinical trials.

From the Department of Medicine, Services of Breast and Gynecological Cancer Medicine and Clinical Immunology, Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, NY; Division of Oncology, University of California, San Francisco; and Pharmacokinetics and Metabolism and Clinical Research, Genentech, Inc, South San Francisco, CA.

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Address reprint requests to José Baselga, MD, Breast and Gynecological Cancer Medicine Service, Memorial Sloan-Kettering Cancer Center, 1275 York Ave, New York, NY 10021-6007.

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We now report the results of a phase II study of multiple-dose intravenous administration of rhuMAb HER2 in patients with metastatic breast cancer. The objectives of this trial were to determine the antitumor activity of rhuMAb HER2 in this patient population, as well as to define further the toxicity profile and pharmacokinetics of rhuMAb HER2.

PATIENTS AND METHODS

Preparation and Humanization of rhuMAb HER2 Antibody

MAb 4D5 was initially derived by immunizing mice with cells that expressed high levels of the HER2 gene product, $p185^{HER2}$.[19] MAb 4D5, directed at the extracellular domain of $p185^{HER2}$ (ECD^{HER2}), inhibits the in vitro growth of breast cancer cells that contain high levels of $p185^{HER2}$.[19,20] rhuMAb HER2 was engineered by inserting the complementarity determining regions of MAb 4D5 into the framework of a consensus human immunoglobulin G_1 (IgG_1).[22] The resulting rhuMAb HER2 has high affinity for $p185^{HER2}$ (Dillohiation constant [K_d] = 0.1 nmol/L), markedly inhibits, in vitro and in human xenografts, the growth of breast cancer cells that contain high levels of $p185^{HER2}$, and induces antibody-dependent cellular cytotoxicity (ADCC).[22,23] rhuMAb HER2 is produced by a genetically engineered Chinese hamster ovary (CHO) cell line, grown in large scale, that secretes rhuMAb HER2 into the culture medium. Antibody is purified from the CHO culture media using standard chromatographic and filtration methods. Each lot of antibody used in this study was assayed to verify identity, purity, and potency, as well as to meet Food and Drug Administration requirements for sterility and safety.

Selection of Patients

Patients eligible for this study were adult women whose metastatic breast carcinomas overexpressed HER2 (see later). All patients had measurable disease, a Karnofsky's performance status of at least 60%, and preserved hematologic, liver, renal, and pulmonary function. Patients with lymphangitic pulmonary metastasis, history of brain metastasis, or bone metastases as the only site of measurable disease were excluded. Chemotherapy or additive hormonal therapy within 3 weeks before study entry (6 weeks for mitomycin or nitrosureas) was not permitted. Informed consent was obtained and documented in writing before study entry.

Tumor expression of HER2 was determined by immunohistochemical analysis, as previously described,[5,6] of a set of thin sections prepared from the patient's paraffin-archived tumor blocks. The primary detecting antibody used was murine MAb 4D5, which has the same complementarity determining regions as rhuMAb HER2. Tumors were considered to overexpress HER2 if at least 25% of tumor cells exhibited characteristic membrane staining for $p185^{HER2}$.

Antibody Administration

The pharmacokinetic goal was to achieve rhuMAb HER2 trough serum concentrations greater than 10 μg/mL, a level associated with optimal inhibition of cell growth in the preclinical model.[22] The optimal dose and schedule of rhuMAb HER2 was based on two prior phase I clinical trials, conducted at University of California, Los Angeles, and Memorial Sloan-Kettering Cancer Center, which had documented dose-dependent pharmacokinetics. In this current trial, rhuMAb HER2 was administered intravenously over a period of 90 minutes in the outpatient setting. Each patient received a loading dose of 250 mg of rhuMAb HER2 on day 0, and beginning on day 7,100 mg weekly for a total of 10 doses. At the completion of this treatment period, patients with stable disease or minor, partial, or complete responses were entered onto a maintenance phase of weekly rhuMAb HER2 administration until disease progression.

Evaluation of Toxicity

Toxicity was scored based on a modified National Cancer Institute common toxicity criteria. Complete blood cell counts, urinalysis, coagulation profile, and hepatic enzyme, renal, and electrolyte studies were performed weekly while on the study.

Pharmacokinetics, Determination of Extracellular Domain of $p185^{HER2}$ Levels, and Antibodies Directed Against rhuMAb HER2

Blood samples for pharmacokinetic analysis were collected just before each treatment with rhuMAb HER2 and within the first hour following the end of each rhuMAb HER2 infusion. Serum concentrations of rhuMAb HER2 were determined in a receptor binding assay that detects binding with ECD^{HER2}. The nominal limit of detection for rhuMAb HER2 in serum samples was 156 ng/mL. The presence of antibodies to rhuMAb HER2 was determined with a bridging-type titer enzyme-linked immunosorbent assay (ELISA). Circulating concentrations of ECD^{HER2} shed by patients' tumors were also determined using an ELISA.[24] The pair of antibodies used for the assay were 7C2 as coat and 2C4 as horseradish peroxidase–conjugated antibody; the lower limit of detection for this assay ranged from 2.8 to 8.3 ng/mL (Baly D, Wong WL, unpublished data, November 1994).

Serum levels of rhuMAb HER2 as a function of time were analyzed for each patient using a one-compartment model. Model parameters (volume and the elimination rate constant [K_e]) were estimated for each patient using a maximum-likelihood estimation procedure.[25] rhuMAb HER2 half-life ($t_{1/2}$) was calculated by dividing ln 2 by K_e.

Tumor Response

Tumor response was determined at the completion of the initial 11-week treatment period. All responses were confirmed by an independent extramural evaluation committee composed of an oncologist and a radiologist. Complete response was defined as the disappearance of all radiographically and/or visually apparent tumor, partial response as a $\geq$ 50% reduction in the sum of the products of the perpendicular diameters of all measurable lesions, minimal response as a $\geq$ 25% and less than 50% reduction in the diameters, stable disease as no change greater than 25% in the size of measurable lesions, and progressive disease as a $\geq$ 25% increase in any measurable lesion or the appearance of any new lesion. Although bone metastases were considered not measurable for response, patients had to have at least stability of bone lesions to be considered responders. Patients who had entered the maintenance phase of the study had tumor responses evaluated every 11 weeks, or earlier if clinically indicated. Time to tumor progression was calculated from the beginning of therapy to progression. Confidence limits for response rates were calculated using the exact method for a single proportion.[26]

Table 1. Patient Characteristics

Characteristic	Patients (N = 46) No.	%
Age, years		
Median	50	
Range	30-65	
Karnofsky performance status		
Median	90	
Range	60-100	
Level of HER2 expression*		
25%-50% cells	7	15.2
> 50% cells	39	84.8
Receptor status		
Estrogen receptor-positive (n = 40)	17	42.5
Progesterone receptor-positive (n = 39)	15	38.5
No. of metastatic sites		
1	16	34.5
2	14	30.4
≥ 3	16	34.5
Dominant site of metastasis		
Viscera	37	80.4
Skeleton	1	2.2
Soft tissues	8	17.4
Prior therapy		
Chemotherapy	45	97.8
Adjuvant chemotherapy	26	56.5
Neoadjuvant chemotherapy	4	8.7
Metastatic disease (no. of regimens)		
None	8	17.4
1	9	19.6
2	9	19.6
> 2	20	43.5
Median	2	
Range	0-7	
Hormonal therapy		
Adjuvant tamoxifen	7	15.2
Metastatic disease	21	45.6

*In percent of tumor cells with cytoplasmic membrane staining.

RESULTS

Patients characteristics are listed in Table 1. A total of 46 patients were enrolled onto the study. Their level of tumor overexpression of HER2 was relatively high, with more than 80% of the tumors having more than half of their cells exhibit positive membrane staining. Our patient population had extensive metastatic disease: 34.5% of patients had three or more metastatic sites. Dominant sites of metastases were visceral in 80% of cases (lung in 18, liver in 13, both liver and lung in five, and ovary in one). Only 17.4% of cases had dominant metastases in soft tissues (skin and lymph nodes) and only one patient had bone as the dominant site of disease. The total number of patients with bone disease was 18 (39%). All but one of the patients had received prior chemotherapy, with 82.6% having received at least one regimen for metastatic disease and 63% having received two or more regimens. Of this latter group, four patients had previously received high-dose chemotherapy with hematopoietic stem-cell support.

Data on rhuMAb HER2 pharmacokinetics are available from 45 patients (Table 2). More than 90% of the examined population (41 patients) had rhuMAb HER2 trough levels above the targeted 10-μg/mL level. The mean serum $t_{1/2}$ of rhuMAb HER2 was 8.3 ± 5.0 days. The rhuMAb HER2 serum $t_{1/2}$ was found to be dependent on the presence of circulating ECD^{HER2} released from the tumor into the serum (Table 2). Representative examples of pharmacokinetics profiles are shown in Fig 1. Figure 1A shows the serum levels of rhuMAb HER2 in a patient with undetectable level of circulating ECD^{HER2}; stable, therapeutic serum levels of the drug were maintained in this patient for more than 1 year. Figure 1B shows the serum levels of rhuMAb HER2 in a patient with high levels of circulating ECD^{HER2}; trough levels of rhuMAb HER2 were consistently below detectable levels throughout the treatment course and until disease progression. Antibodies against rhuMAb HER2 (human antihuman antibodies [HAHA]) were not detected in any patients.

Treatment with rhuMAb HER2 was remarkably well tolerated. Of a total of 768 administrations of rhuMAb HER2, only 11 events occurred that were considered to be related to the use of the antibody (Table 3). Fever and chills occurred on five occasions after the first administration of rhuMAb HER2. The fever lasted less than 8 hours in all cases and did not recur on subsequent administrations of the antibody. Three patients experienced chest pain in areas of tumor involvement shortly after the infusion of the first dose of rhuMAb HER2; in one case this required an overnight hospital admission for pain control. The pain did not recur on successive administrations of the antibody. None of the patients whose cancer regression met the formal criteria for complete or partial response had pain at a tumor site after administration of rhuMAb HER2.

The number of patients assessable for treatment response on evaluation day 77 was 43. Three patients were not assessable for response. One had a bacteremic infection of an intravenous catheter that required prolonged administration of antibiotics, which precluded treatment

Table 2. ECD^{HER2}-Dependent Pharmacokinetics of rhuMAb HER2

N	Patient Group	rhuMAb HER2 $t_{1/2}$ (days)
45	All patients	8.3 ± 5.0
40	Circulating ECD^{HER2} < 500 ng/mL	9.1 ± 4.7
5	Circulating ECD^{HER2} > 500 ng/mL	1.8 ± 1.0

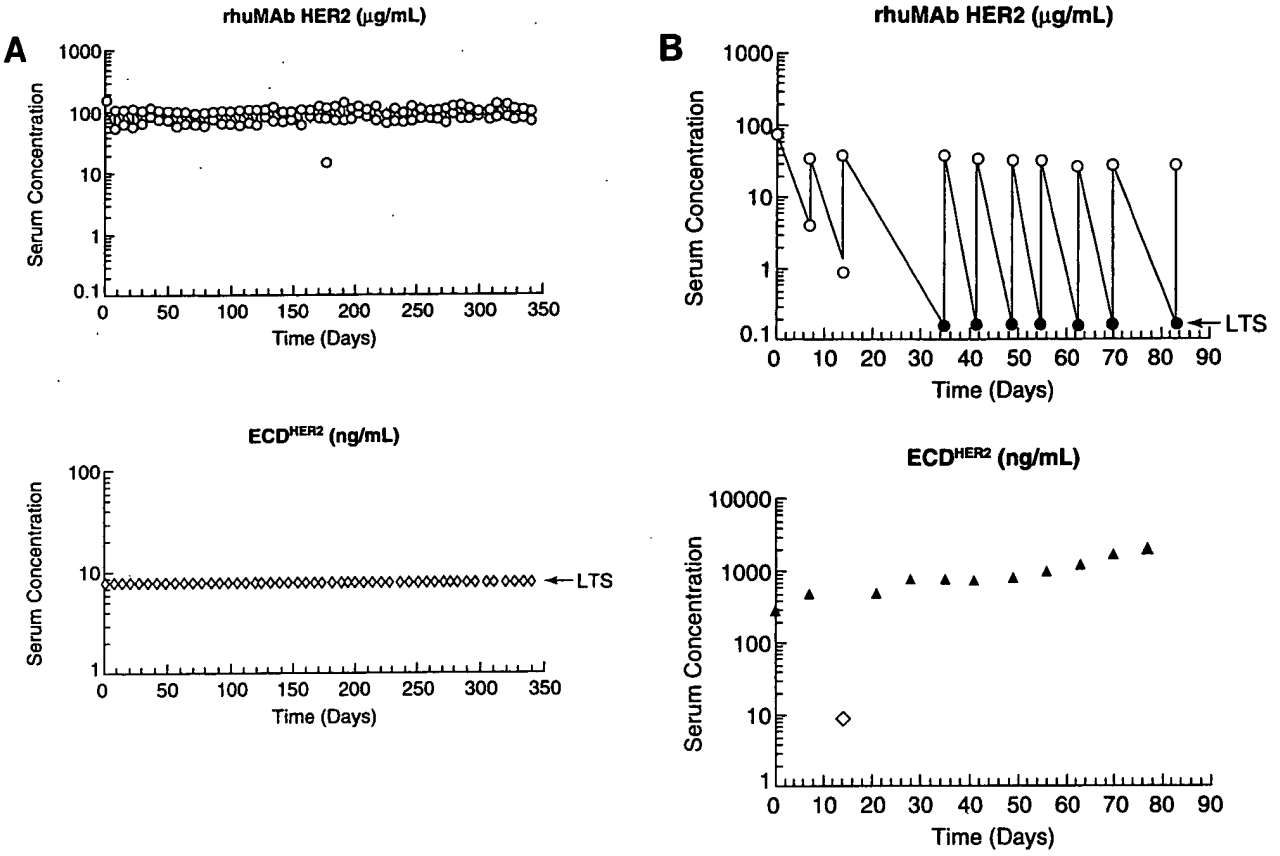

Fig 1. Effect of serum ECD^{HER2} on rhuMAb HER2 pharmacokinetics. Stable serum levels of rhuMAb HER2 in a patient with absence of ECD^{HER2} (A) v suboptimal rhuMAb HER2 serum levels in a patient with high ECD^{HER2} (B). Note that log scales on the Y-axis describing the serum ECD^{HER2} differ among charts. LTS, less than lowest assay standard. (○) observed rhuMAb HER2 serum concentration; (●) LTS for rhuMAb HER2 serum concentration; (▲) ECD^{HER2} serum concentration; (◇) LTS for ECD^{HER2} serum concentration.

with rhuMAb HER2. A second declined to continue on the study for personal reasons. The third died of congestive heart failure associated with prior doxorubicin treatment. Among 43 assessable patients, 5 had tumor responses: one patient had a complete remission and four had partial remissions. Therefore, the overall response rate (complete plus partial responses) for assessable patients is 11.6% (95% confidence interval, 4.36 to 25.9). Details of responses are listed in Tables 4 and 5, and examples of the responses are shown in Fig 2.

Table 3. rhuMAb HER2-Related Toxicity

Toxicity	Moderate (grade 2)	Severe (grade 3)
Fever and chills	5	
Pain at tumor site	2	1
Diarrhea	2	
Nausea and emesis	1	

NOTE. In number of events of a total of 768 administrations.

Two patients had minor responses and 14 patients had stable disease at day 77. These patients entered a maintenance phase of weekly antibody administration until progression of disease. The median time to progression for the patients with either minor or stable disease was 5.1 months. An additional patient had a greater than 50% reduction in the size of the metastatic disease on her mediastinum and chest wall after 2 weeks of treatment. While the duration of response was greater than 4 weeks, by evaluation day 77 the lesion had begun to regrow from the size of maximal response to therapy. Per protocol guidelines, this patient was therefore considered not to have had a response to therapy, but rather progression of disease.

DISCUSSION

During the last decade, overexpression of the HER2 gene has been shown to play an important role in the pathogenesis and poor prognosis of breast cancer. As a

Table 4. Response Rate Obtained With rhuMAb HER2 in 43 Assessable Patients

Response	No. of Patients	%
Complete response	1	2.3
Partial response	4	9.3
Overall response	5	11.6
Minor response	2	4.6
Stable disease	14	32.6
Progression of disease	22	51.2

consequence, strategies directed at interference with HER2 expression or the function of its protein, $p185^{HER2}$, have been anticipated to have therapeutic value. Extensive preclinical studies have shown that certain MAbs directed against $p185^{HER2}$ can inhibit growth of HER2-overexpressing tumor cells.[15-19] This study provides the first clinical evidence of the antitumor activity of one of these agents, rhuMAb HER2.

Of 43 patients with $p185^{HER2}$-positive tumors assessable for response after treatment with rhuMAb HER2, five experienced a complete or partial remission, for an overall response rate of 11.6%. One additional patient had a greater than 50% shrinking of her cancer that lasted more than 1 month, but was not considered a responder by our protocol definition. The objective antitumor responses observed were of clinical importance, since two patients had regression of cancers in the liver and one patient achieved a pathologically-proven complete response of chest wall disease, which has persisted for 24 months. Our patients were selected to have many sites of metastatic involvement, one of the most dire prognostic characteristics regarding response to therapy. This selection was the consequence of the rule that patients with disease involving only bone were ineligible for accrual, because bone is the solitary site of initial metastatic involvement in up to 60% of cases.[27] It is reasonable to hypothesize that the percentage of patients who show objective tumor regression to rhuMAb HER2 will be higher when patients with less extensive breast cancer are treated, since laboratory studies have shown that the response to antireceptor antibodies is greater with lower tumor burden.[28] It would be also of interest to analyze the response rate to rhuMAb HER2 in a patient population with no prior chemotherapy for stage IV disease, since prior experience has shown that untreated patients usually respond better to new anticancer drugs.[29]

Another important point about the probability of response to rhuMAb HER2 concerns the observation that 37% of patients achieved minimal responses or stable disease. In the laboratory, rhuMAb HER2 or the parent antibody 4D5 has been noted to be cytostatic, which causes growth arrest, rather than cytocidal, which causes cell death. In clinical trials of many anticancer drugs, particularly chemotherapy, the achievement of stable disease is not considered a reliable measure of anticancer activity. However, with rhuMAb HER2, stable disease may be an authentic reflection of the biologic action of the drug, which differs markedly from conventional anticancer agents. The unusually long durations of minimal responses and stable disease seen in our trial may relate to this distinction. These data are specially interesting in light of the absence of significant toxicity observed here, for in a setting in which palliation is a main objective, quality of life while on treatment should be a main end point.

The dose and schedule of rhuMAb HER2 administration used in this protocol provided adequate serum concentrations in all patients, except in those with circulating levels of tumor-shed ECD^{HER2} at serum concentrations ≥

Table 5. Characteristics of Patients Who Achieved a Response to Treatment

Patient No.	HER2*	Site of Metastatic Disease	Prior Systemic Therapy	Best Response	Duration of Response (months)
1	3+	Chest wall	Doxorubicin	Complete response†	> 24
2	3+	Liver	Doxorubicin, mitoxantrone, paclitaxel	Partial response	6.7
3	2+	Mediastinum	CMFVP, doxorubicin, tamoxifen, paclitaxel	Partial response	7.7
4	3+	Liver + retroperitoneal lymph nodes + bone	CMF, docetaxel	Partial response	1
5	2+	Chest wall	Paclitaxel	Partial response	3.4

Abbreviations: CMFVP, cyclophosphamide, methotrexate, fluorouracil, vincristine, and prednisone; CMF, cyclophosphamide, methotrexate, and fluorouracil.

*By immunohistochemistry: 2+, 25% to 50% of tumor cells with cytoplasmic membrane staining; 3+, > 50% of tumor cells with cytoplasmic membrane staining.

†Patient's complete response was pathologically proven with several biopsies at tumor site. Patient bone scan, head, thoracic, abdominal, and pelvic computed tomographic scans are negative.

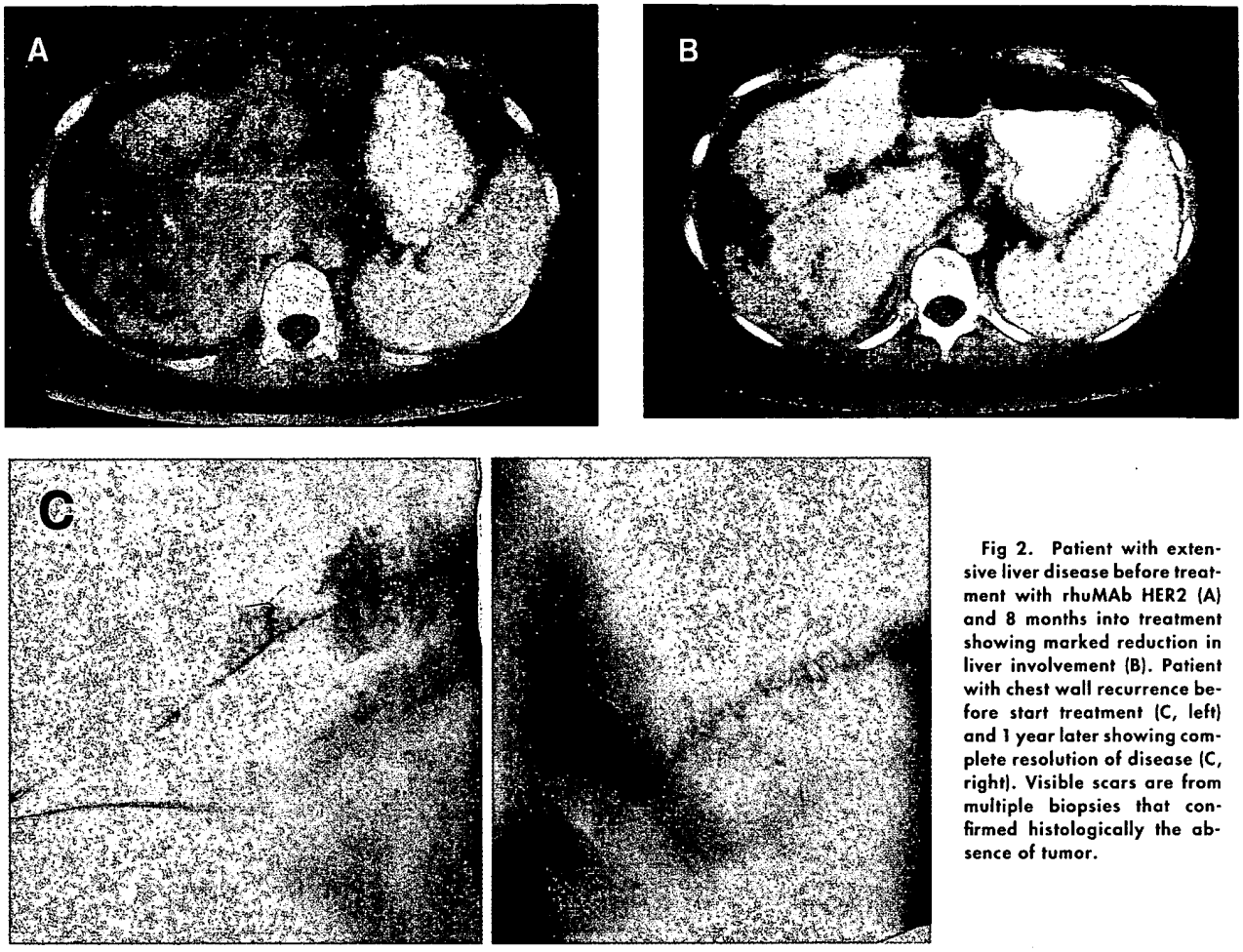

Fig 2. Patient with extensive liver disease before treatment with rhuMAb HER2 (A) and 8 months into treatment showing marked reduction in liver involvement (B). Patient with chest wall recurrence before start treatment (C, left) and 1 year later showing complete resolution of disease (C, right). Visible scars are from multiple biopsies that confirmed histologically the absence of tumor.

500 ng/mL. ECD^{HER2} is known to be released by some breast cancer cells that overexpress HER2,[30-32] and elevated ECD^{HER2} serum levels have been previously reported in patients with breast cancer.[31,33,34] The most likely explanation for the short serum $t_{1/2}$ values and subtherapeutic trough levels of rhuMAb HER2 in this group of patients is that in the presence of ECD^{HER2} in the serum, antigen-antibody complexes form and are rapidly cleared from the circulation. Of interest, no anticancer responses were observed in the group of patients with serum concentrations of $ECD^{HER2} \geq 500$ ng/mL. Hence, the interpretation of results of future trials of agents that bind to or exert their function through $p185^{HER2}$ should take ECD^{HER2} release from the tumors into account; at present, patients with high levels of ECD^{HER2} should continue to participate in these studies.

There are several possible mechanisms, not mutually exclusive, that could explain the clinical results observed. An important fact is that rhuMAb HER2 induces a marked downregulation of $p185^{HER2}$.[19] Antibody-induced downregulation of $p185^{HER2}$ has been shown to induce reversion of the transformed phenotype in HER2-transformed cells.[14] By a similar mechanism, the continuous exposure to rhuMAb HER2 at adequate concentrations achieved in our trial could be reversing the malignant phenotype of the clinical cancers by downregulating their level of $p185^{HER2}$. Another possibility is that the known partial agonistic effects of rhuMAb HER2[35] could result in the activation of a signal transduction pathway that leads to inhibition of tumor-cell proliferation. Both of these potential antitumor mechanisms would require, in addition to receptor expression, intact receptor function. Little is known about the functional status of $p185^{HER2}$ in breast tumor specimens, but it is conceivable that not all overexpressing tumors have functional receptors. In support of this view is the observation that HER2-overexpressing tumor-cell lines that are not growth-inhibited by anti-$p185^{HER2}$ antibodies have been described and well charac-

terized[36]; it is noteworthy that some of these antibody-resistant tumor cells also overexpressed truncated forms of ECD^{HER2}. Furthermore, in vitro studies suggest that those breast cancer cell lines that have the highest basal level of p185^{HER2} phosphorylation are the most growth-inhibited by anti-p185^{HER2} antibodies.[20] If this were the case in the clinic, the recently produced antiserum that specifically recognizes only overexpressed tyrosine-phosphorylated p185^{HER2} might prove useful in predicting the subset of p185^{HER2}-positive tumors most likely to respond to rhuMAb HER2.[8]

Another possible mechanism of action concerns the observation that rhuMAb HER2 is a potent inducer of ADCC.[22] However, while this immune-mediated mechanism might play a role in the observed clinical responses, ADCC is obviously not involved in the pronounced growth-inhibitory effects of the antibody in vitro.

The observed activity of rhuMAb HER2 against advanced breast cancers that overexpress HER2 provides the first clinical evidence that anti–growth factor receptor-directed strategies may be useful in the treatment of human breast cancer. Therefore, continued research with this agent and other HER2-targeted treatment strategies appears warranted. The response to rhuMAb HER2 in a less heavily pretreated population and in those with less extensive metastatic disease would be of interest since both parameters have historically correlated with a higher response to drugs,[29] and this same principle may apply to antibody-based therapy.[37] In preclinical studies, both in vitro and in xenografts, rhuMAb HER2 markedly potentiated the antitumor effects of several chemotherapeutic agents, including cisplatin, doxorubicin, and paclitaxel,[23,38] without increasing their toxicity. Laboratory studies of the mechanism of this effect and clinical trials of such combination therapy are currently in progress.

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REFERENCES

1. Aaronson SA: Growth factors and cancer. Science 254:1146-1153, 1991

2. Coussens L, Yang-Feng TL, Liao Y-C, et al: Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. Science 230:1132-1139, 1985

3. Akiyama T, Sudo C, Ogawara H, et al: The product of the human c-erbB-2 gene: A 185-kilodalton glycoprotein with tyrosine kinase activity. Science 232:1644-1646, 1986

4. Stern DF, Hefferman PA, Weinberg RA: p185, a product of the *neu* proto-oncogene, is a receptorlike protein associated with tyrosine kinase activity. Mol Cell Biol 6:1729-1740, 1986

5. Slamon DJ, Godolphin W, Jones LA, et al: Studies of the HER-2/*neu* proto-oncogene in human breast and ovarian cancer. Science 244:707-712, 1989

6. Slamon DJ, Clark GM, Wong SG, et al: Human breast cancer: Correlation of relapse and survival with amplification of the HER2/*neu* oncogene. Science 235:177-182, 1987

7. Ravdin PV, Chamness GC: The c-erbB-2 proto-oncogene as a prognostic and predictive marker in breast cancer: A paradigm for the development of other macromolecular markers—A review. Gene 159:19-27, 1995

8. Hynes NE, Stern DF: The biology of *erB-2/neu*/HER-2 and its role in cancer. Biochim Biophys Acta 1198:165-184, 1994

9. Hudziak RM, Schlessinger J, Ullrich A: Increased expression of the putative growth factor receptor p185^{HER2} causes transformation and tumorigenesis of NIH 3T3 cells. Proc Natl Acad Sci USA 84:7159-7163, 1987

10. Di Fiore PP, Pierce JH, Kraus MH, et al: *erb*B-2 is a potent oncogene when overexpressed in NIH-3T3 cells. Science 237:178-182, 1987

11. Guy CT, Webster MA, Schaller M, et al: Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc Natl Acad Sci USA 89:10578-10582, 1992

12. Gusterson BA, Machin LG, Gullick WJ, et al: Immunohistochemical distribution of c-erbB-2 in infiltrating and in situ breast cancer. Int J Cancer 42:842-845, 1988

13. van de Vijver MJ, Peterse JL, Mooi WJ, et al: *NEU*-protein overexpression in breast cancer—Association with comedo-type ductal carcinoma in situ and limited prognostic value in stage II breast cancer. N Engl J Med 319:1239-1245, 1988

14. Drebin JA, Link VC, Stern DF, et al: Down-modulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies. Cell 41:695-706, 1985

15. McKenzie SJ, Marks PJ, Lam T, et al: Generation and characterization of monoclonal antibodies specific for the human *neu* oncogene product, p185. Oncogene 4:543-548, 1989

16. Stancovski I, Hurwitz E, Leitner D, et al: Mechanistic aspects of the opposing effects of monoclonal antibodies to the *erb*B-2 receptor on tumor growth. Proc Natl Acad Sci USA 88:8691-8695, 1991

17. Hancock MC, Langton BC, Chan T, et al: A monoclonal antibody against the c-erbB-2 protein enhances the cytotoxicity of cis-diamminedichloroplatinum against human breast and ovarian tumor cell lines. Cancer Res 51:4575-4580, 1991

18. Harwerth IM, Wels W, Schlegel J, et al: Monoclonal antibodies directed to the erbB-2 receptor inhibit in vivo tumour cell growth. Br J Cancer 68:1140-1145, 1993

19. Hudziak RM, Lewis GD, Winget M, et al: p185^{HER2} monoclonal antibody has antiproliferative effects in vitro and sensitizes human breast tumor cells to tumor necrosis factor. Mol Cell Biol 9:1165-1172, 1989

20. Lewis GD, Figari I, Fendly B, et al: Differential responses of human tumor cell lines to anti-p185^{HER2} monoclonal antibodies. Cancer Immunol Immunother 37:255-263, 1993

21. Shepard HM, Lewis GD, Sarup JC, et al: Monoclonal antibody therapy of human cancer: Taking the HER2 protooncogene to the clinic (review). J Clin Immunol 11:117-127, 1991

22. Carter P, Presta L, Gorman CM, et al: Humanization of an anti-p185HER2 antibody for human cancer therapy. Proc Natl Acad Sci USA 89:4285-4289, 1992

23. Baselga J, Norton L, Coplan K, et al: Anti-HER2 humanized monoclonal antibody alone and in combination with chemotherapy against human breast carcinoma xenografts. Proc Am Soc Clin Oncol 13:63, 1994 (abstr 53)

24. Sias PE, Kotts CE, Vetterlein D, et al: ELISA for quantitation of the extracellular domain of p185^{HER2} in biological fluids. J Immunol Methods 132:73-80, 1990

25. D'Argenio DZ, Schumitzky A: ADAPT II User's Guide (ed 2). Los Angeles, CA, Biomedical Simulations Resource, University of Southern California, 1990

26. Fleiss JL: Statistical Methods for Rates and Proportions (ed 2). New York, NY, Wiley, 1981, pp 13-17

27. Hayes DF, Kaplan WD: Evaluation of patients following primary therapy, in Harris JR, Hellman S, Kinne DW (eds): Breast Diseases (ed 2). Philadelphia, PA, Lippincott, 1991, pp 505-525

28. Fan Z, Baselga J, Masui H, et al: Antitumor effect of anti-epidermal growth factor receptor monoclonal antibodies plus cis-diamminedichloroplatinum on well established A431 cell xenografts. Cancer Res 53:4637-4642, 1993

29. Norton L: Salvage chemotherapy of breast cancer. Semin Oncol 21:19-24, 1994

30. Zabrecky JR, Lam T, McKenzie SJ, et al: The extracellular domain of p185/neu is released from the surface of human breast carcinoma cells, SK-BR-3. J Biol Chem 266:1716-1720, 1991

31. Pupa SM, Menard S, Morelli D, et al: The extracellular domain of the c-erbB-2 oncoprotein is released from tumor cells by proteolytic cleavage. Oncogene 8:2917-2923, 1993

32. Lin YJ, Clinton GM: A soluble protein related to the HER-2 proto-oncogene product is released from human breast carcinoma cells. Oncogene 6:639-643, 1991

33. Hayes DF, Cirrincione C, Carney W, et al: Elevated circulating HER2/neu related protein (NRP) is associated with poor survival in patients with metastatic breast cancer. Proc Am Soc Clin Oncol 12:58, 1993 (abstr 35)

34. Kandl H, Seymour L, Bezwoda WR: Soluble c-erbB-2 fragment in serum correlates with disease stage and predicts for shortened survival in patients with early-stage and advanced breast cancer. Br J Cancer 70:739-742, 1994

35. Scott GK, Dodson JM, Montgomery PA, et al: p185HER2 signal transduction in breast cancer cells. J Biol Chem 266:14300-14305, 1991

36. Scott GK, Robles R, Park JW, et al: A truncated intracellular HER2/neu receptor produced by alternative RNA processing affects growth of human carcinoma cells. Mol Cell Biol 13:2247-2257, 1993

37. Riethmuller G, Schneider-Gadicke E, Schlimok G, et al: Randomised trial of monoclonal antibody for adjuvant therapy of resected Dukes' C colorectal carcinoma. Lancet 343:1177-1183, 1994

38. Pietras RJ, Fendly BM, Chazin VR, et al: Antibody to HER-2/*neu* receptor blocks DNA repair after cisplatin in human breast and ovarian cancer cells. Oncogene 9:1829-1838, 1994